# Cell Cycle Regulation by Phosphatases

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# Declaration

I, Molly Diane Godfrey, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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## Publications Arising from this Thesis

**Godfrey M.**, Kuilman T., Uhlmann F. (2015). Nur1 Dephosphorylation Confers Positive Feedback to Mitotic Exit Phosphatase Activation in Budding Yeast. *PLoS Genet* 11(1) (2015)

Kuilman T., Maiolica A., **Godfrey M.**, Scheidel N., Aebersold R. and Uhlmann F. (2015). Identification of Cdk targets that control cytokinesis. *The EMBO Journal* 34(1) 81–96 (2015)

## Abstract

In eukaryotes, the timing of cell cycle events and transitions is controlled via substrate phosphorylation and dephosphorylation, by kinases and phosphatases. In budding yeast, the ratio of Cdk kinase/Cdk-opposing phosphatase activity regulates mitotic exit by controlling the timing of substrate dephosphorylation in a quantitative manner.

The main mitotic exit phosphatase in budding yeast is Cdc14. In interphase and early mitosis, Cdc14 is sequestered in the nucleolus through inhibitory binding to Net1, from which it is released in early anaphase. At this time, Net1 is phosphorylated by Cdk (thus relieving its inhibition of Cdc14), an event promoted by proteins of the Cdc14 Early Anaphase Release (FEAR) network. Later in anaphase, the Mitotic Exit Network (MEN) signaling cascade maintains Cdc14 release. We have yet to understand how Cdc14 activity can increase in early anaphase, while Cdk activity, that is required for Net1 phosphorylation, decreases and the MEN is not yet active. I show that the nuclear rim protein Nur1 interacts with Net1 and, in its Cdk phosphorylated form, inhibits Cdc14 release. Cdc14 dephosphorylates Nur1 in early anaphase, relieving the inhibition and promoting further Cdc14 release. Nur1 dephosphorylation thus describes a positive feedback loop in Cdc14 phosphatase activation during mitotic exit.

The importance of Cdk-opposing phosphatase activity in mitotic exit is established, but it is postulated that similarly, Cdk-opposing phosphatase activity in interphase could be an important element in a quantitative model for cell cycle progression. In budding yeast, PP2A, Cdc14, and PP1 are candidates for such a phosphatase. In the absence of $PP2A^{Cdc55}$, global interphase Cdk phosphorylation levels are elevated, independently of PP2A's role in regulating Cdk tyrosine phosphorylation. Strikingly, absence of $PP2A^{Cdc55}$ leads to a specific increase in threonine over serine Cdk phosphorylation. I have also identified specific Cdk targets whose phosphorylation is advanced and increased in this case. This indicates that $PP2A^{Cdc55}$ may be directly opposing Cdk phosphorylation events throughout interphase, from G1 until the onset of mitosis, thus forming a part of a mechanism that sets Cdk thresholds for cell cycle progression. The roles of PP1 and Cdc14 in interphase remain as yet undetermined.

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# Abbreviations

| | |
|---|---|
| Ab | Antibody |
| Acm | APC/C(Cdh1) modulator |
| APC/C | Anaphase promoting Complex/Cyclosome |
| ARS | Autonomously Replicating Sequence |
| Ase | Anaphase spindle elongation |
| Ask | Associated with Spindles and Kinetochores |
| Bub | Budding Uninhibited by Benzimidazole |
| Cdk | Cyclin Dependent Kinase |
| Cdc | Cell Division Cycle |
| Cdh | Cdc20 Homolog |
| CPC | Chromosome passenger complex |
| Dam | Duo1 And Mps1 interacting |
| Dbf | Dumb Bell Former |
| Dbp | Dead Box Protein |
| dNTP | Deoxynucleotide triphosphate |
| DNA | Deoxyribonucleic Acid |
| DTT | Dithiothreitol |
| ECL | Enhanced chemiluminescence |
| EDTA | Ethylene diamine tetraacetic acid |
| FACS | Fluoresence activated cell sorting |
| FEAR | Cdc fourteen early anaphase release |
| Fin | Filaments in between nuclei |

| | |
|---|---|
| G1/2 | Gap phase 1/2 |
| GAL | Galactose inducible promoter |
| GFP | Green fluorescent protein |
| Glc | Glycogen |
| h | hours |
| HA | Hemagglutinin |
| HRP | Horseradish peroxidase |
| Ipl | Increase in Ploidy |
| Kb | Kilobase |
| kDa | kiloDalton |
| Ndd | Nuclear Division Defective |
| Net | Nucleolar silencing Establishing factor & Telophase regulator |
| nm | nanometre |
| nM | nanoMolar |
| NTS | Non-transcribed Spacer |
| M | Mitosis |
| Mb | Megabase |
| MEF | Mouse Embryonic Fibroblast |
| MEN | Mitotic Exit Network |
| min | minute |
| Mms | Methyl MethaneSulfonate sensitivity |
| mM | milliMolar |
| ml | millilitre |
| Mps | MonoPolar Spindle |

| | |
|---|---|
| OA | Okadaic Acid |
| Orc | Origin Recognition Complex |
| PCR | Polymerase Chain Reaction |
| PEG | PolyEthylene Glycol |
| PI | Propidium Iodide |
| PP | Protein Phosphatase |
| PPh | Protein Phosphatase |
| rDNA | Ribosomal Deoxyribonucleic Acid |
| RNA | Ribonucleic Acid |
| RNAse | Ribonuclease |
| rRNA | Ribosomal Ribonucleic Acid |
| rpm | revolutions per minute |
| Rts | Rox Three Suppressor |
| s | seconds |
| S | Synthesis |
| SILAC | Stable Isotope LAbelling in Culture |
| Sli | Synthetically Lethal with Ipl1 |
| Smc | Structural Maintenance of Chromosomes |
| SDS | Sodium Dodecyl Sulfate |
| SDS-PAGE | Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis |
| Sld2 | Synthetically Lethal with Dpb11-1 |
| Slk | Synthetic Lethal Kar3p |
| ssDNA | single stranded DNA |
| ts | temperature sensitive |

| | |
|---|---|
| wt | wild type |
| YNB | Yeast Nitrogen Base |
| YP | Yeast Peptone |
| YPD | Yeast Peptone Dextrose |
| µl | microlitre |
| µm | micrometre |

# Chapter 1. Introduction

## 1.1 Cell division is a fundamental and conserved process

"The dream of every cell is to become two cells", according to the molecular biologist and Nobel-prize winner Francois Jacob (Monod and Wainhouse, 1971), and this "dream" is the fundamental priniciple driving all living organisms, the essential condition for life itself. Since the cell theory was formulated in the 19$^{th}$ century, we have tried to understand how cell division works, which external and internal factors influence how, when, and why, cells divide. Although we can now claim to have a good overall grasp of the control of cell division in organisms ranging from the simple to the complex, from bacteria to yeast, frogs to fish, birds to mammals, a detailed and complete understanding of the molecular mechanisms involved remains beyond our reach.

In this thesis, using the baker's yeast *Saccharomyces cerevisiae* as a model organism, I have aimed to provide my own contribution to the field of cell division research, building on previous knowledge to provide an ever-clearer picture of how cells reproduce.

### 1.1.1 The eukaryotic cell cycle

In order to reproduce, a "parent" cell first needs to grow in size and duplicate its contents, and then divide cellular components equally between two newly-formed distinct entities known as "daughter" cells. The tightly regulated series of events occuring during these processes forms the cell cycle, which can be divided into several phases. Fundamental to this process is the need to replicate the DNA (in the form of chromosomes), and then separate the newly replicated genetic material equally and accurately between the daughter cells. These processes occur respectively during the "synthesis" or S phase and at mitosis or M phase.

Mitosis can be further subdivided into metaphase, anaphase and telophase, and is followed by cytokinesis, during which the daughter cells separate. These phases are

separated by gap phases (G1 and G2) required for cellular growth and the replication of organelles (Fig. 1.1). The G1, S and G2 phases separating successive mitoses are collectively known as interphase (Forsburg and Nurse, 1990; Morgan, 2007).

These basic events, and the order they occur in, are conserved in all eukaryotic cells. The details of cell cycle regulation and time spent in gap phases vary. Further, there are variations in the symmetry of cell division and whether or not the nuclear envelope breaks down (open vs. closed mitoses) between different organisms, and in the case of the former, within organisms.

### 1.1.2 The cell cycle of the budding yeast *Saccharomyces cerevisiae*

*Saccharomyces cerevisiae*, a simple fungal unicellular organism, has emerged over the last 40 years as an advantageous model system to study cell cycle regulation (Hartwell, 1974; Forsburg and Nurse, 1990). Basic cell cycle events and the way these are regulated are conserved between yeast and other eukaryotic systems. Indeed, the cell cycle is controlled, to a large degree, by structural and functional homologues of the same basic set of proteins in humans and in yeast (Morgan, 2007). Their speed of replication, and amenability to genetic manipulation, the availability of well-developed techniques and their low cost of use, as well as their simplicity, make yeast a good model organism and basic research tool.

The particulars of the budding yeast cell cycle involve asymmetric cell growth and division, and a closed mitosis, with no breakdown of the nuclear envelope (Fig. 1.1). The genome, around 12 Mb long and spread over 16 chromosomes, is replicated in S phase, with sister chromatids undergoing a process of condensation throughout G2 and metaphase, and being segregated in anaphase. Spindle pole bodies duplicate in early S phase and the bipolar spindle forms at the G2/M transition, aligning itself across the nucleus and elongating throughout mitosis in order to drag sister chromatids apart. The spindle then breaks down in telophase.

At 25°C, the doubling time for wild type budding yeast cells growing exponentially under optimal conditions is around 90 minutes. Cells spend the majority of this time

alternating between S phase (approximately 20-30 minutes) and mitosis (approximately 45-50 minutes, more or less equally divided between metaphase and anaphase, with telophase and cytokinesis being very brief).

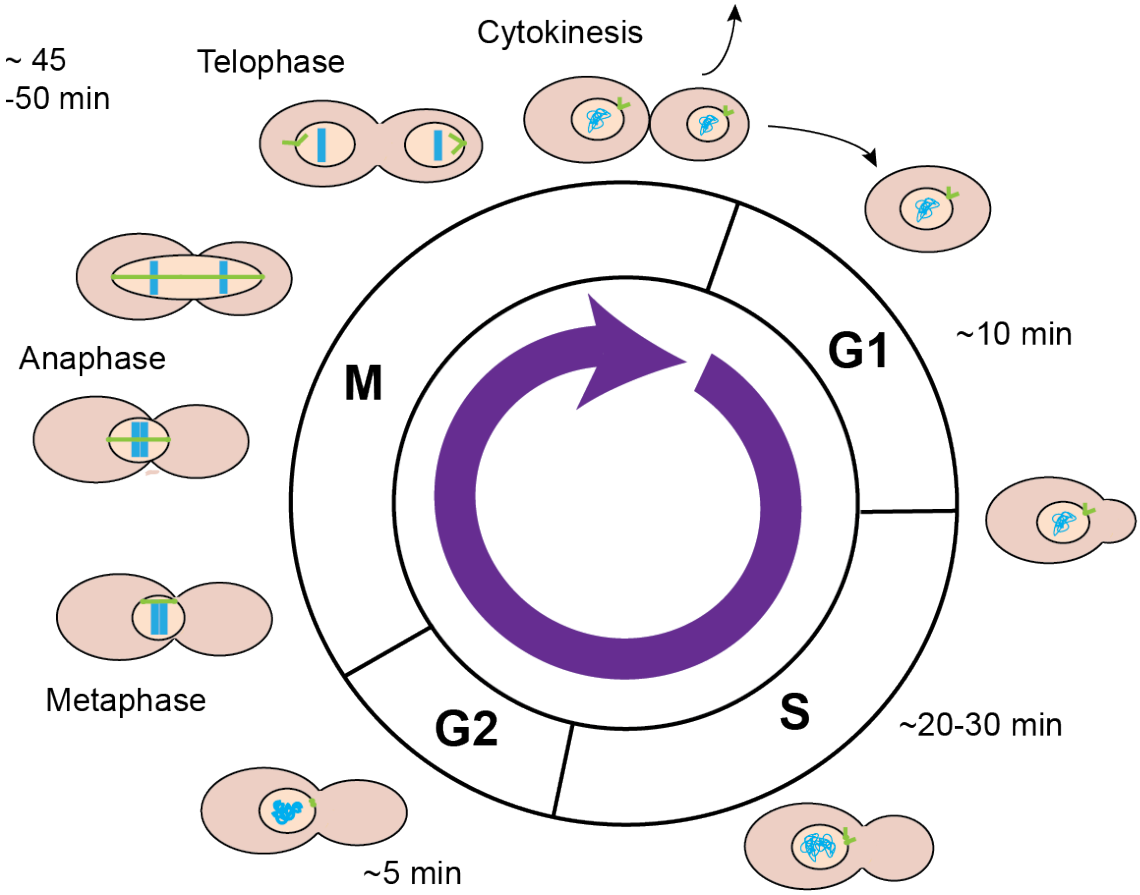



**Figure 1.1 – The budding yeast cell cycle**
The eukaryotic cell cycle is composed of S phase, during which chromosomes are replicated, and M phase, during which they are separated and at the end of which cell division occurs. S phase and M phase are separated by periods of cell growth, known as gap phases (G1 and G2). In budding yeast, cell division is asymmetric and the daughter cell is formed by budding from the mother cell. The nuclear envelope does not break down during mitosis. DNA in the form of uncondensed (G1-S-G2) and condensed (M) chromosomes is represented in blue, inside the nucleus, and the spindle and spindle pole bodies are in green. The approximate times spent in each phase of the cell cycle in cells growing exponentially at 25°C is indicated.

As in other eukaryotes, two proteins, both ring shaped complexes composed of members of the SMC family of proteins, provide the structural integrity to condensed, mitotic chromosomes – Condensin and Cohesin. Condensin (and to some extent Cohesin) is responsible for condensing the chromosomes, providing them with enough rigidity and identity to allow them to be organised into distinct sister chromatids (Thadani, Uhlmann and Heeger, 2012). Cohesin holds the replicated sister chromatids together from the end of S phase until anaphase, when it is cleaved, allowing them to segregate to opposite poles of the cell (Haering, Löwe, Hochwagen and Nasmyth, 2002; Uhlmann, Lottspeich and Nasmyth, 1999; Marston, 2014).

### 1.1.3 Cell cycle checkpoints

Cells must ensure that their DNA is accurately copied and chromosomes equally partitioned between two cells in order to preserve ploidy and integrity of the genome. Therefore S phase and mitosis must be temporally regulated to occur only once each cell cycle, and any errors in replication or segregation need to be rapidly detected. Moreover, the cell needs to make sure that external conditions are favourable before commiting to cell replication. This is accomplished via checkpoints throughout the cell cycle, the principles and players behind which are highy conserved (Hartwell and Weinert, 1989; Elledge, 1996; Morgan, 2007).

The first checkpoint regulates entry into the cell cyle, and is known as the G1 or restriction checkpoint in higher eukayotes, or START, in yeast (Cross, 1995; Pardee, 1974). At this point, cells decide whether or not to commit to a round of replication, a decision dependent on exogenous influences. In yeast, commitment depends mainly on enviromental factors, whereas in human cells it is more likely to be triggered by growth factors.

DNA integrity is monitored both before replication, at the G1/S transition, and before mitosis, at the G2/M transition – the cells "check" that the chromosomes have been properly replicated before entering into mitosis (Gerald, Benjamin and Kron, 2002). Budding yeast also have a morphogenesis checkpoint, where bud formation and correct

cell polarity is monitored (Lew and Reed, 1995). Finally, the "mitotic" or "spindle assembly" checkpoint ensures that the chromosomes are correctly bi-oriented and under tension from the spindle, ensuring accurate chromosome segregation (Musacchio and Salmon, 2007; May and Hardwick, 2006).

## 1.2 Cell cycle control by Cyclin-Cdk complexes

### 1.2.1 Cell cycle progression is controlled by Cdc28 associated with a series of cell cycle stage-specific cyclins

Cyclin-dependent kinases (Cdk's) are the master regulators of the eukaryotic cell cycle. In complex with regulatory molecules known as Cyclins, which provide substrate specificity and stimulation of kinase activity, they control the ordering of cell cycle events. Cdk's are proline-directed serine/threonine phosphatases, phosphorylating the Cdk consensus site motif S/TP(xR/K), with the +3 lysine/arginine preferred but not essential (Holmes and Solomon, 1996). They control cell cycle progression by phosphorylating, and thereby activating/inactivating, stabilising/destabilising, target proteins, actions that are necessary for the completion of cell cycle events in the right order. In budding yeast, there is only one Cdk, known as Cdc28, and 9 different Cyclins, which can be subdivided into three groups: G1 cyclins, Cln1,2,3; S phase cyclins; Clb5,6; and M-phase Cyclins, Clb1,2,3,4 (Bloom and Cross, 2007a; Morgan, 2007). Cdc28 associates with the different cyclins at the appropriate cell cycle stages, allowing the thus formed Cyclin-Cdk complex to phosphorylate the correct target proteins.

G1 Cyclin-Cdk complexes are necessary for licensing the duplication of the spindle pole bodies, promoting emergence of the bud and transcription of the S-phase cyclins. S phase Cyclin-Cdk complexes promote DNA replication and transcription of the mitotic cyclins, which themselves allow for spindle formation, and promote the G2/M transition and other early mitotic events. Mitotic cyclins also inhibit mitotic exit events from occurring (Bloom and Cross, 2007a) (Fig. 1.2). They are degraded at the metaphase-to-anaphase transition, at which point Cdk activity drops dramatically in order to allow for mitotic exit to occur.

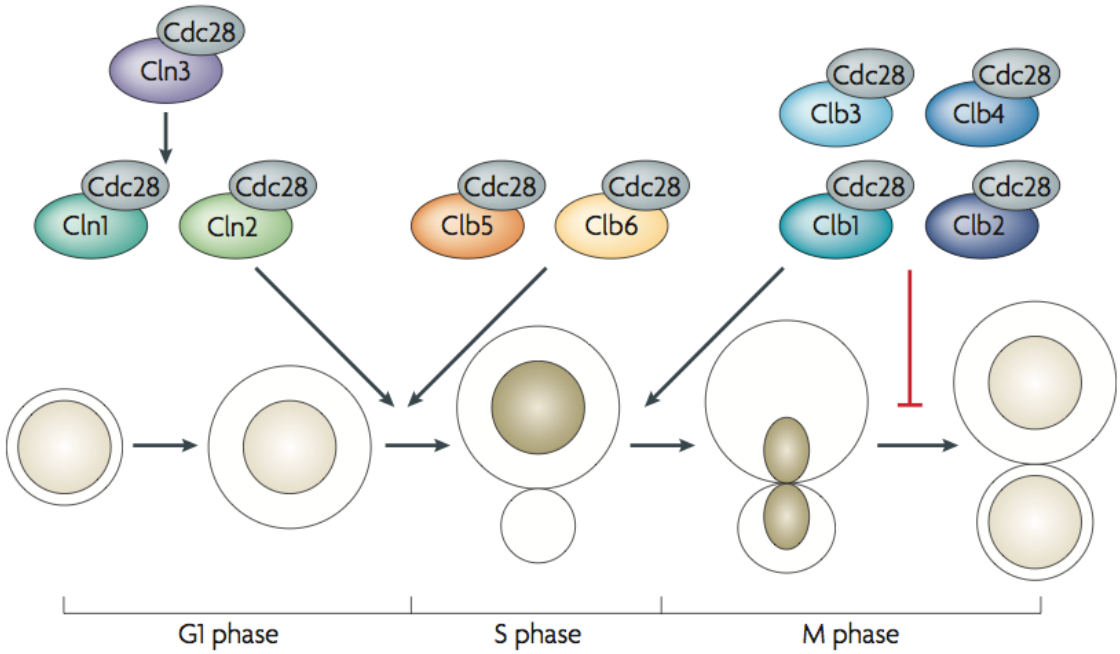



**Figure 1.2 – Budding yeast Cyclin-Cdk complexes in the cell cycle**
Budding yeast cyclins associate with a single Cdk (Cdc28) at different cell cycle stages, during which they promote specific cell cycle events and transitions. From (Bloom and Cross, 2007a).

Although this is a simpler system than that present in higher eukaryotes, where multiple Cdk's exist as well as multiple cyclins, the principle remains the same: cell cycle stages are associated with specific Cyclin-Cdk complexes. As such, it was long assumed that the ordered progression through cell cycle stages was controlled by the specificity of substrates for the different Cyclin-Cdk complexes.

Indeed, supporting this hypothesis, it has been demonstrated in mammalian systems that G1 Cyclin-Cdk complexes have a biochemical preference for a small number of substrates (Kitagawa et al., 1996). In this same system, the existence of an S-phase specific recognition motif termed the RxL peptide was discovered, which is recognised by a hydrophobic patch on human S-phase cyclin (Adams et al., 1996). Strikingly, the role of the hydrophobic patch/RxL motif in conferring a biochemical preference of S phase Cdk-Cyclins for S-phase substrates was confirmed in budding yeast, in a large-scale survey of 150 Cdk substrates (Loog and Morgan, 2005). Further, in budding yeast, an "LP motif" has been identified as a docking site within the G1 Cln1/2-Cdk target

Sic1, promoting phosphorylation by the latter (Kõivomägi et al., 2011). The same study identified several targets that seem to be specific for respectively G1, S and M cyclins. An attempt to pinpoint specific substrates of the different Cyclin-Cdk complexes by screening *Xenopus leavis* cDNA libraries resulted in the identification of around 40 substrates each of Cyclin B-Cdk1 and Cyclin E-Cdk2 complexes (Errico et al., 2010). Similarly, *in vivo* pull-downs of Cyclin-Cdks in complex with their substrates at the different cell cycle stages led to the identification of a range of G1, S or M phase Cdk targets (Pagliuca et al., 2011).

### 1.2.2 A quantitative model for cell cycle control

However, despite this apparent "Cyclin-specificity", cyclins themselves are mainly interchangeable. As such, in budding yeast, cells deleted for all of the B-type cyclins (*clb1-6Δ*), usually inviable, can be rescued by the ectopic expression of a single mitotic cyclin (Clb1) (Schwob, Böhm, Mendenhall and Nasmyth, 1994) .

Further, cells lacking any two out of three of the G1 cyclins are also viable. In fact, cells lacking all three G1 cyclins are, likewise, viable when their deletion is combined with that of the Cdk inhibitor Sic1, a specific inhibitor of Clb-Cyclin complexes. In the absence of Sic1, Clb-Cdk complexes are able to initiate Start and promote the G1/S transition, indicating that Cln and Clb type cyclins are substitutable (Tyers, 1996).

Cells deleted for the S-phase cyclins Clb5 and 6 have a delay in S-phase and the initiation of DNA replication, which is not rescued by the expression of the main mitotic cyclin Clb2 from the Clb5 promoter, supporting the hypothesis that cyclin specificity is the main driver for correct cell cycle progression (Cross, Yuste-Rojas, Gray and Jacobson, 1999). However, it has now been shown that deletion of the Cdk inhibitor Swe1, which has a strong preference for inhibiting Cdk in complex with the mitotic cyclin Clb2, enables Clb2, expressed under control of the Clb5 promoter, to initiate DNA replication with similar efficiency to Clb5. Indeed, in a strain lacking all of the other Clb's and Swe1, the early expressed Clb2-Cdk1 is able to phosphorylate all essential Clb-Cdk substrates with virtually wild-type timing (Hu and Aparicio, 2005).

Similarly, in animal cells, only one, mitotic, Cdk-Cyclin complex is essential – Cdk1-Cyclin B (out of at least 10 Cdk's and at least fourteen Cyclins) (Santamaría et al., 2007; Pagliuca et al., 2011; Kalaszczynska et al., 2009).

Despite the identification of specific substrates for Cyclin B-Cdk1 and Cyclin E-Cdk2 in *X. leavis*, further study of the *in vitro* substrate specificity of these Cyclin-Cdk complexes indicates that the great majority (90%) of substrates were phosphorylated with similar affinity by both kinases. *In silico* analysis could not find any defining factor between one group of substrates and the other (Errico et al., 2010). Further, although cell cycle specific substrates of different Cyclin-Cdk complexes were identified by Pagliuca *et al,* with a range of substrates interacting only with one Cyclin-Cdk complex, this could be due to the presence of each specific complex at different cell cycle stages and with different localisations, combined with the fact that the pull-downs were carried out *in vivo* at these specific stages (Pagliuca et al., 2011).

In addition to this, evidence from fission yeast, in which a single (mitotic) Cyclin-Cdk complex, Cdc13-Cdc2 (Cyclin B-Cdk1), is able to substitute for all others, indicates that perhaps cyclin specificity is not, after all, the main driver for the temporal organisation of cell cycle stages. As such, it was shown some 20 years ago that the oscillations in activity of the Cdc13-Cdc2 complex, in the absence of G1 and S-phase cyclins, could promote both DNA replication and mitosis, and in the correct order (Fisher and Nurse, 1996). This finding led Stern and Nurse to propose their cell cycle model in which rising levels of activity of the Cdk complex, rather than cyclin subunit identity, control the timing of cell cycle events (Stern and Nurse, 1996) (Fig. 1.3A).

This model, for quantitative control of the cell cycle by Cdk's, was a seminal contribution to the field of cell cycle studies. Its strength was reasserted in a series of more recent experiments in fission yeast, in which a genetically engineered Cyclin-Cdk complex, composed of a fusion of Cdc2 and Cdc13 (expressed under the Cdc13 promoter), and a mutation rendering Cdc2 sensitive to chemical inhibition by the ATP analogue 1-NMPP1, was used. This complex is controlled through addition of the inhibitor in a dose-dependent manner, and is naturally degraded at the

metaphase/anaphase transition (due to the fusion with the B-type cyclin Cdc13). Low levels of activity trigger S phase, and high activity causes mitotic entry, whether S phase has been completed or not. Changing Cyclin-Cdk levels is sufficient to overcome cell cycle checkpoints as well as alter the directionality of the cell cycle (Fig. 1.3B).

In further support of this theory, a quantitative model for the ordering of mitotic events also holds true. In budding yeast, it was demonstrated through titration of the levels of Clb2-Cdc28 that mitotic events require increasing concentrations of this complex with relation to their time of incidence. As such, different thresholds of Cdc28-Clb2 activity are required for spindle formation in metaphase and for spindle elongation at anaphase. Further, increasing Clb2-Cdc28 concentration to higher than endogenous levels is sufficient to accelerate mitosis (Oikonomou and Cross, 2011). This "Cdk threshold" model has also been validated in mammalian cells (Deibler and Kirschner, 2010). Exit from mitosis, in this quantitative model for the cell cycle, depends on the rapid drop in Cdk activity resulting from Cyclin degradation (Wäsch and Cross, 2002) (Fig. 1.3A).

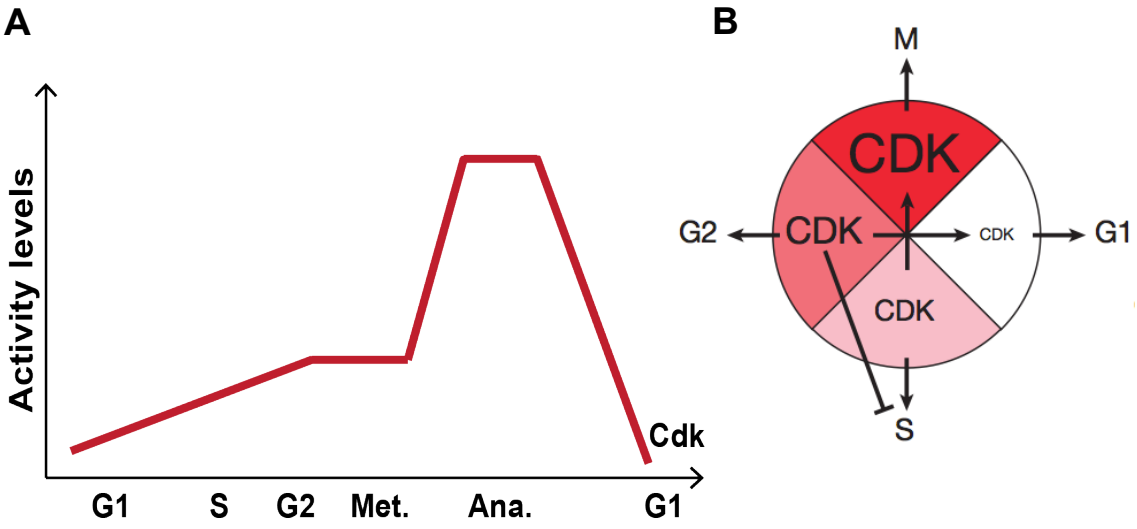



**Figure 1.3 – A quantitative model for the cell cycle**
**A**. A quantitative model for cell cycle control, where rising Cdk levels control progression through the different phases of the cell cycle, as well as through mitosis. Adapted from (Stern and Nurse, 1996) **B.** The cell cycle lacks core directionality – S phase does not need to have been completed for M phase to occur, and cells reciprocally do not need to have exited mitosis in order to perform S phase again. The only dependence is on the quantitative levels of qualitatively the same Cdc13-Cdc2 complex (Adapted from (Coudreuse and Nurse, 2010)).

## 1.3 A focus on budding yeast mitotic exit

### 1.3.1 Key events in budding yeast mitotic exit

At anaphase onset, Cdk activity begins to drop as the Anaphase Promoting Complex/Cyclosome (APC/C) targets cyclins for degradation (Wäsch and Cross, 2002). The activation of the APC/C itself is dependent upon the inactivation of the Spindle Assembly Checkpoint or Mitotic Checkpoint, which monitors that the chromosomes are correctly attached to the mitotic spindle via microtubule/kinetochore interactions. APC is activated by phosphorylation by Clb-Cdk, with Clb-Cdk complexes thus promoting their own inactivation once they have reached their highest threshold of activity (Rahal and Amon, 2008).

This triggers the sequence of events composing mitotic exit. One of the first proteins to be degraded by the APC, in complex with its Cdc20 regulatory subunit, is the securin Pds1. Pds1 degradation allows the activation of the protease Separase (Esp1 in budding yeast), which then goes on to cleave the Cohesin ring (Uhlmann, Lottspeich and Nasmyth, 1999). Cohesion between the sister chromatids is therefore dissolved, which then allows them to be pulled by the now rapidly elongating spindle to opposite ends of the cell.

In budding yeast, Cdk activity persists in early anaphase. Although Clb5 is rapidly degraded upon anaphase onset, leading to an initial drop in Cdk activity, only a small fraction of Clb2 is degraded upon the activation of $APC^{Cdc20}$ (Nasmyth, Shirayama, Tóth and Gálová, 1999; Bäumer, Braus and Irniger, 2000). The bulk of Clb2 degradation in fact begins slightly later, when Cdc20 is replaced by Cdh1 – this protein being phosphorylated and inhibited from associating with APC until late anaphase (Jaspersen, Charles and Morgan, 1999). After this, the rest of Clb2 is degraded, Cdk activity falls further, the chromosomes decondense and the spindle disassembles in telophase.

### 1.3.2 The Cdk-opposing phosphatase Cdc14 is the major player in budding yeast mitotic exit

The fall in Cdk activity is not sufficient, by itself, to drive mitotic exit. Instead, it is accompanied by a rise in the activity of Cdc14, a Cdk-counteracting phosphatase. Cdc14 was first identified as cell cycle regulator of which mutants arrested in late anaphase by Lee Hartwell in 1971 (Culotti and Hartwell, 1971). However, its role as the major player in budding yeast mitotic exit was not recognised for another 25 years, at which point it became apparent that Cdc14 triggers mitotic exit by directly dephosphorylating Cdk substrates (Visintin et al., 1998).

One of the main targets of Cdc14 is the Cdk inhibitor Sic1, which is stabilised upon its dephosphorylation. Another important target of Cdc14 is Cdh1, allowing for full activation of the APC (Jaspersen, Charles and Morgan, 1999). This allows for a dual regulation of Cdk activity – both by degradation of Cyclins and inhibition by Sic1. Indeed, it is the concomitant degradation of cyclins (causing the drop in Cdk activity) along with the re-accumulation and reactivation of Sic1, that make the M/G1 transition irreversible (López-Avilés, Kapuy, Novak and Uhlmann, 2009). Aside from that, Cdc14 is essential for the regulation of all mitotic exit events up to and including cytokinesis, dephosphorylating many if not all Clb-Cdk substrates during this cell cycle phase. As such, it not only brings about mitotic exit but also "resets" the cell cycle for the next G1.

Amongst others, its targets include Ask1, Fin1 and Sli15, their dephosphorylation necessary for spindle stability. It also targets Orc6, in order to relicense replication in the next cell cycle, as well as the replication factors Sld2 and Dpb2. Finally, it can also dephosphorylate proteins involved in cytokinesis, such as Inn1, to promote accurate bud neck formation (Higuchi and Uhlmann, 2005; Bouchoux and Uhlmann, 2011; Mirchenko and Uhlmann, 2010; Woodbury and Morgan, 2007; Bloom and Cross, 2007b; Sanchez-Diaz, Nkosi, Murray and Labib, 2012).

## 1.4 The activity of Cdc14 is stringently regulated

Given the importance of Cdc14 for mitotic exit, its high affinity for Cdk substrates, and its Cdk-opposing activity, it needs to be regulated so that it is only present and active in the cell during a short time-window. As such, during most of the cell cycle, Cdc14 is kept sequestered and inactive in the nucleolus through inhibitory binding to the rDNA-associated protein Net1 (also known as Cfi1) (Traverso et al., 2001; Visintin, Hwang and Amon, 1999; Shou et al., 1999). Net1 likely inhibits Cdc14 by occluding the active site of the phosphatase, acting as a highly specific competitive inhibitor in *in vitro* experiments (Traverso et al., 2001).

Net1 deletion causes slow cell growth, with delays in events throughout the cell cycle – notably bud formation, DNA replication, and mitotic entry. These delays are presumably due to Cdc14 being active towards interphase Cdk targets at this time (Visintin, Hwang and Amon, 1999). Indeed, whether or not *net1Δ* is actually viable has produced contradictory results, with this deletion having been identified as lethal in the *S. cerevisiae* S288C background in a genome-wide gene deletion screen (Giaever et al., 2002). Cdc14 localisation can be de-regulated by using a $CDC14^{TAB6\text{-}1}$ allele, which binds Net1 less strongly (Shou et al., 2001). Although, alone, this allele does not have a strong phenotype, it is lethal together with *clb5Δ* – cells to fail to complete DNA replication in this case (Bloom and Cross, 2007b). This is likely because the combination of lower than usual Cdk activity with higher than usual Cdk-opposing phosphatase activity does not allow for the timely phosphorylation of the Cdk substrates necessary for DNA replication. This indicates the importance of proper regulation of Cdc14 activity.

Cdc14 is activated and released from the nucleolus after phosphorylation of Net1 by both Cdk and Polo kinase (Cdc5). Phosphorylation of Net1 leads to reduced affinity for Cdc14; *in vitro*, phosphorylated Net1 is no longer able to inhibit Cdc14 (Azzam et al., 2004; Yoshida and Toh-e, 2002; Shou et al., 2002). Net1 phosphorylation, and therefore Cdc14 release, is prevented until early anaphase by the phosphatase $PP2A^{Cdc55}$, which keeps Net1 hypo-phosphorylated (Queralt, Lehane, Novak and Uhlmann, 2006).

In fact, release of Cdc14 from the nucleolus, achieved through the regulation of the phosphorylation state of Net1, occurs in two waves, depending on the actions of two successive signalling cascades, the FEAR (Cdc14 Early Anaphase Release) and MEN (Mitotic Exit Network) pathways.

### 1.4.1 The FEAR pathway for Cdc14 release promotes early anaphase events

Cdc14 release is initially activated by the actions of $APC^{Cdc20}$, and the degradation of securin. As such, once Esp1 (separase) has been released from Pds1 (securin), it has been shown to have a role other than that of cleaving cohesin; that of inhibiting $PP2A^{Cdc55}$(Queralt et al., 2006).

The inhibition of $PP2A^{Cdc55}$ by Separase is brought about by the action of Separase and its interactor Slk19 upon the Cdc55 regulators Zds1 and Zds2, which regulate the localisation of Cdc55 in anaphase and prevent it from interacting with Net1(Queralt and Uhlmann, 2008; Rossio and Yoshida, 2011). Further, the proteins Fob1 and Spo12 also contribute, together, to the activation of Cdc14 in early anaphase, although how they do so is not yet fully understood (Stegmeier, Visintin and Amon, 2002; Stegmeier et al., 2004). Along with Cdc5 and Clb-Cdk, all of the above proteins thus form a part of the FEAR pathway for Cdc14 release in early anaphase (Rock and Amon, 2009) (Fig. 1.4).

FEAR pathway released Cdc14 is predominantly restricted to the nucleus, and is necessary for coordinating a range of anaphase events in this cell cycle compartment. These range from promoting rDNA condensation and segregation, through controlling nuclear positioning, to spindle stabilisation and elongation, and positioning of the spindle midzone (D'amours, Stegmeier and Amon, 2004; Sullivan, Higuchi, Katis and Uhlmann, 2004; Mirchenko and Uhlmann, 2010; Higuchi and Uhlmann, 2005; Ross and Cohen-Fix, 2004; Wang, Yong-Gonzalez and Strunnikov, 2004; Woodbury and Morgan, 2007). The timely release of this phosphatase, synchronised with separase

activation, allows for the accurate coordination of chromosome segregation with the initiation of mitotic exit events.

However, the FEAR pathway for Cdc14 is neither essential, nor enough to allow for the completion of mitotic exit. Absence of FEAR pathway components (barring separase, securin and PP2A, which have many other roles in the cell) is not lethal, and simply results in delayed Cdc14 release, and concomitantly delayed mitotic exit.

### 1.4.2 The MEN pathway for Cdc14 release promotes late mitotic events and cytokinesis

As such, a second signalling pathway is necessary for the sustained release of Cdc14 in late anaphase. Indeed, early Cdc14 release is promoted by Cdk phosphorylation of Net1. With $APC^{Cdh1}$ active in later anaphase, rapidly degrading the mitotic cyclins and thus causing a fast decline in Cdk activity, Net1 phosphorylation can no longer be maintained by the kinase.

To maintain Cdc14 release after this point, declining Cdk activity and rising Cdc14 activity result in the activation of the G-protein coupled signaling cascade known as the MEN, consisting of the GTPase Tem1, its regulators Lte1 and Bub2/ Bfa1, and its downstream kinases Cdc15 and Dbf2/Mob1 (Fig. 1.4) (Shou et al., 1999; Jaspersen, Charles, Tinker-Kulberg and Morgan, 1998) . Cdc15 is dephosphorylated in mid-anaphase by Cdc14, dephosphorylation that is thought to contribute to its activation (Jaspersen and Morgan, 2000). Similarly, Mob1 is phosphorylated and inhibited by Cdk and dephosphorylated and activated by Cdc14 (König, Maekawa and Schiebel, 2010). Thus Cdc14 activates its own sustained release. Cdc5, the only Cdc14 regulator that is a member of both the FEAR and MEN, inhibits Bub2/Bfa1, themselves inhibitory regulators of Tem1 (Hu et al., 2001). MEN pathway released Cdc14 is necessary for the completion of mitosis and cytokinesis. Indeed, mutants for Tem1, Cdc15 or Dbf2 arrest in telophase with fully segregated chromosomes but long spindles (Shirayama, Matsui and Toh-E, 1994; Culotti and Hartwell, 1971; Toyn and Johnston, 1994; Sanchez-Diaz et al., 2012).

How the MEN is responsible for maintaining Net1's phosphorylation despite declining Cdk activity is unclear, although it is likely that the MEN kinases Dbf2, Mob1 and Cdc15 have the potential to target Net1, as well as Cdc5. Moreover, how Cdc14 release is sustained while Cdk activity declines between anaphase onset and MEN activation has remained poorly understood.

At the M/G1 transition, (through the actions of Cdc14 itself, as well as those of $PP2A^{Cdc55}$, whose inhibition ends) Net1 becomes dephosphorylated, which in turn promotes the re-sequestration of Cdc14, in time for the following cell cycle.

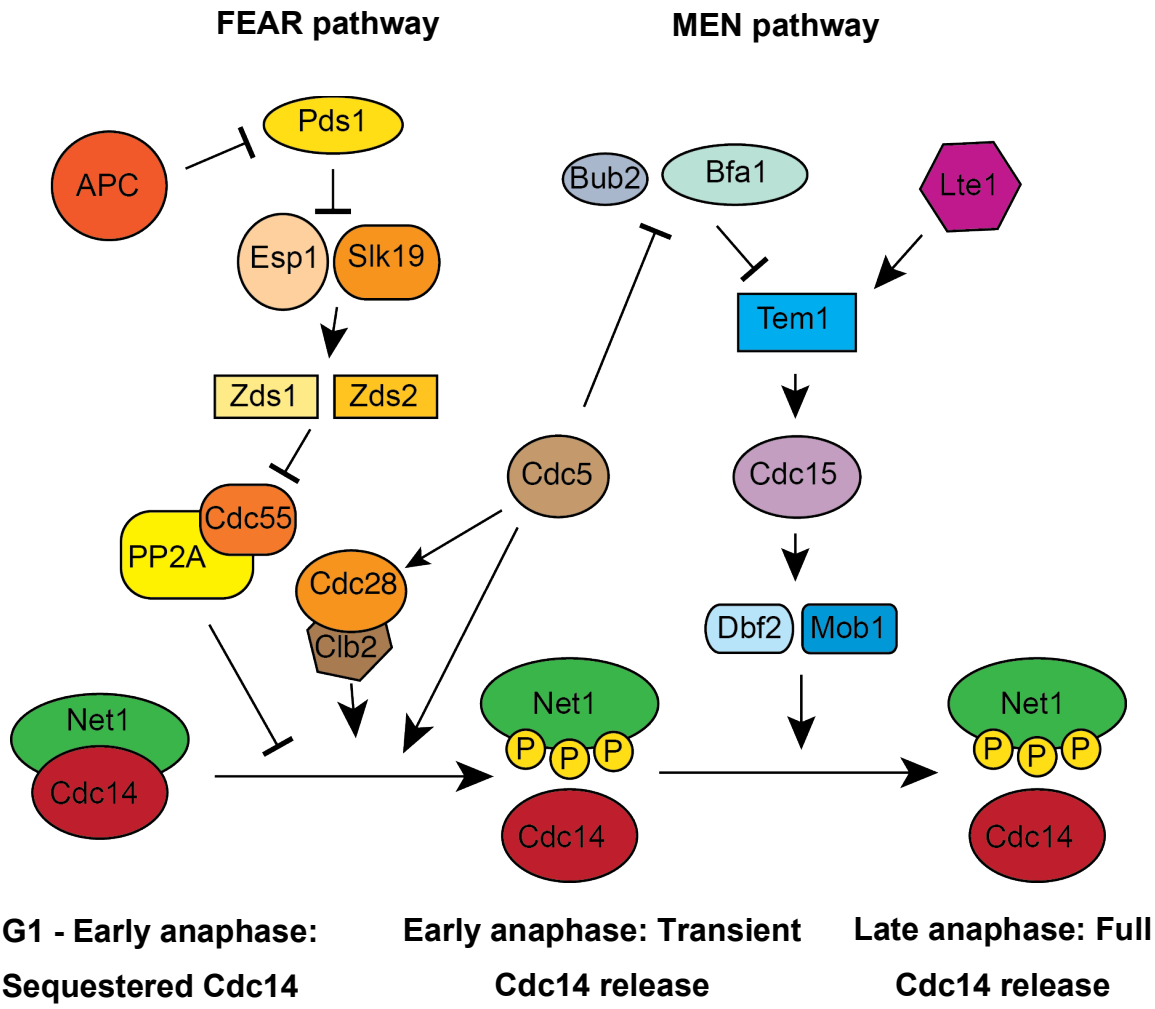



**Figure 1.4 – The FEAR and MEN pathways cooperate to release Cdc14 in two waves**
An initial and transient wave of Cdc14 is released in early anaphase through the actions of FEAR pathway components, necessary for a coordinating a range of anaphase events. Sustained Cdc14 release occurs later in anaphase, through the actions of the MEN, and is necessary for the completion of mitotic exit and cytokinesis.

## 1.5 Cdc14 is required for rDNA condensation and segregation

One of the main roles for FEAR pathway released Cdc14 is in promoting the timely and accurate segregation of the rDNA.

### 1.5.1 The rDNA is the last region of the genome to segregate

The rDNA is a unique region of the genome. It is composed of 150-200 identical repeats, each 9.1 kb in length, tandemly arrayed on the long arm of Chromosome XII, (making up around 70% of the long arm of the DNA on chromosome XII). Each rDNA repeat comprises two genes, the 35S rRNA gene (the product of which is later processed to generate mature 18S, 5.8S and 25S rRNA transcripts) and the 5S rRNA gene. Further, there are several cis elements within each repeat – two nontranscribed spacer regions (NTS1 and NTS2), within which are situated, respectively, the origin of DNA replication (ARS), and the replication fork barrier (RFB) (Brewer and Fangman, 1988; Linskens and Huberman, 1988) (Fig. 1.5). Together, the rDNA repeats and their associated proteins make up the complex macromolecular structure known as the nucleolus – a factory for the production of ribosomal RNA and a region important for cell cycle regulation through its sequestration of Cdc14.

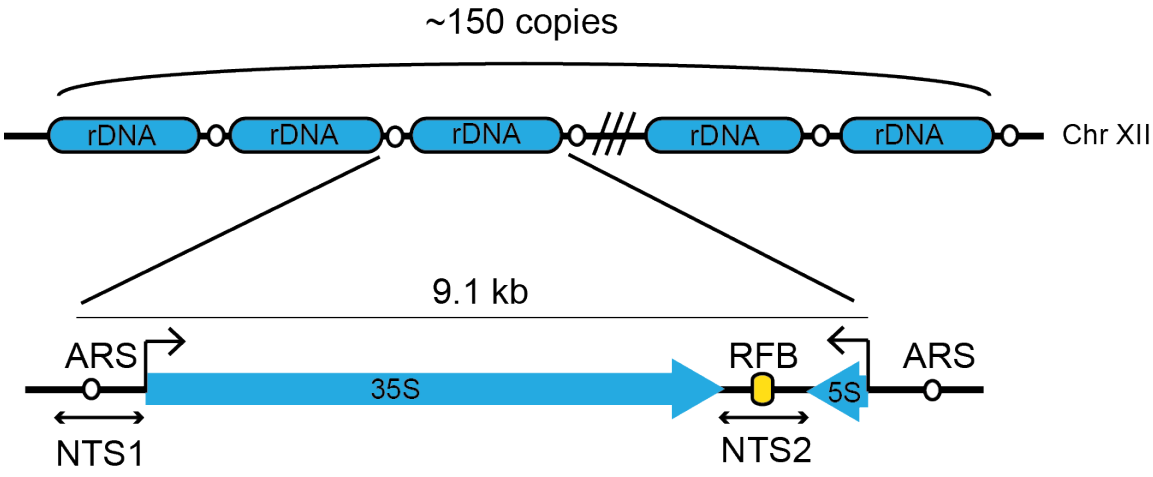



**Figure 1.5 – Structure of the budding yeast rDNA repeats**
150 to 200 repeats of the rDNA are arrayed on the long arm of Chr XII. Within each repeat are the genes for the 35S and 5S rRNA precursors, an origin of replication (ARS) and a replication fork barrier (RFB), where replication terminates.

The rDNA is also unique in that its behaviour during the cell cycle is different to that of the rest of the chromosomes. As such, it has long been observed that the nucleolus is the last region of the genome to segregate (Granot and Snyder, 1991; Sullivan and Uhlmann, 2003). In contrast to the rest of the chromosomes, artificially induced cohesin cleavage (in a metaphase arrested state, in the absence of separase activity but in the presence of the externally expressed TEV protease) is not sufficient to allow the segregation of the rDNA (Sullivan et al., 2004). In fact, Cdc14 activity in early anaphase is required for the segregation of the rDNA; *cdc14-ts* mutants arrest in mid-anaphase with the bulk of the DNA segregated but not the rDNA. In contrast, *cdc15-2* mutants, in which the MEN pathway, but not the FEAR pathway, is defective, show two distinct nucleolar masses, situated at opposite ends of the cell – correctly segregated rDNA {(D'amours, Stegmeier and Amon, 2004; Sullivan et al., 2004). Further, a requirement for the non-cohesin directed activity of separase in early anaphase – that of activating the FEAR pathway – has been shown (Sullivan et al., 2004).

### 1.5.2 Cdc14 is required for Condensin recruitment to the rDNA

One of the known requirements of Cdc14 for rDNA segregation is in fact the ability of this phosphatase to promote condensation of the rDNA. As such, the rDNA is less condensed in the absence of Cdc14 activity than it is in wild-type cells. Further, the rDNA locus, as well as segregating after the rest of the genome, only finishes compacting in late anaphase, again after the rest of the genome has already segregated – the former likely a consequence of the latter (Machin, 2005). Condensin is required for late compaction of the rDNA locus, with recruitment of condensin being impaired in Cdc14 mutants (Sullivan et al., 2004; D'amours, Stegmeier and Amon, 2004; Wang, Yong-Gonzalez and Strunnikov, 2004; Machin, 2005). Indeed, the majority of the Condensin in budding yeast mitosis localises to the rDNA, and Condensin mutants segregate the bulk of the chromosomes but not the nucleolus, reminiscent to that which occurs in Cdc14 mutants (Freeman, 2000). Condensin promotes decatenation of the rDNA locus as well as compacting it, probably through stimulating the activity of Topoisomerase II, further promoting its timely and accurate segregation (D'Ambrosio, Kelly, Shirahige and Uhlmann, 2008).

However, how Cdc14 activity and Condensin recruitment to the rDNA are linked is not yet fully clear. On the one hand, it seems logical that Condensin itself should be the Cdc14 target involved, with dephosphorylation controlling its recruitment to the rDNA. However, a direct link between Cdc14 and Condensin has yet to be confirmed. It has also been suggested that Cdc14 prevents transcription at the rDNA locus during anaphase by inhibiting RNA polymerase I, facilitating the access of Condensin to the locus (Clemente-Blanco et al., 2009). However, it has been previously and extensively shown that rDNA transcription does not vary in a cell cycle dependent manner. As such, the bulk of cellular rRNA increases exponentially during the cell cycle, and rRNA synthesis rates and r-protein synthesis rates per unit amount of RNA remain constant throughout all cell cycle stages (Elliott and McLaughlin, 1979; Elliott, Warner and McLaughlin, 1979; Elliott and McLaughlin, 1983).

## 1.6 A role for phosphatases in the quantitative model for the cell cycle

### 1.6.1 A quantitative model for mitotic exit

Through the successive actions of the FEAR and MEN, a situation arises where Cdc14 activity is gradually rising throughout anaphase, with Clb-Cdk activity concomitantly declining. From a qualitative point of view, however, the same phosphatase, with the same activity, is present in the cell in early anaphase and late anaphase, albeit at different levels. This has led people to ask how this same phosphatase can drive the ordered dephosphorylation of different substrates, and ensure that mitotic exit events happen in the correct order? Indeed, it seems logical that spindle elongation should occur before spindle disassembly, and that chromosome segregation happen before chromosome decondensation. How is this achieved?

In examining how the timing of dephosphorylation by Cdc14 is controlled, Bouchoux and Uhlmann have recently proposed an answer to this question. They examined the catalytic efficiencies of “early” and “late” dephosphorylated substrates, for both Cdc14 and Clb2-Cdk, by *in vitro* reconstitution of the system. There is, overall, little

correlation between the catalytic efficiencies of Clb2-Cdc28 for these substrates and their timing of dephosphorylation. Significantly, the opposite result was noted for Cdc14. Further, an even greater correlation was recorded between the ratios of catalytic efficiencies of phosphatase versus kinase. They were also able to conclude that the timing of dephosphorylation was not directly related to cyclin specificity (i.e. substrates of S phase cyclins being dephosphorylated before those of M phase cyclins, for instance Orc6, a substrate for Clb5, is dephosphorylated late in mitotic exit) or number of phosphosites on the target protein (Bouchoux and Uhlmann, 2011).

In fact, that the ratio of Cdc14 to Cdk activity should control timing of target substrate dephosphorylation during mitosis seems logical. As such, at any time in mitotic exit, substrates are subjected to both phosphorylation by Cdk and dephosphorylation by Cdc14. It is only when the activity of Cdc14 can "win" over that of Cdk, that the target protein can be dephosphorylated. How Cdc14 possesses higher affinity for certain substrates over others remains to be determined.

In this way, a quantitative mechanism for control of ordered substrate dephosphorylation during mitotic exit is revealed, complementing Stern and Nurse's quantitative model for the control of ordered substrate phosphorylation before and during mitotic entry (Fig. 1.6).

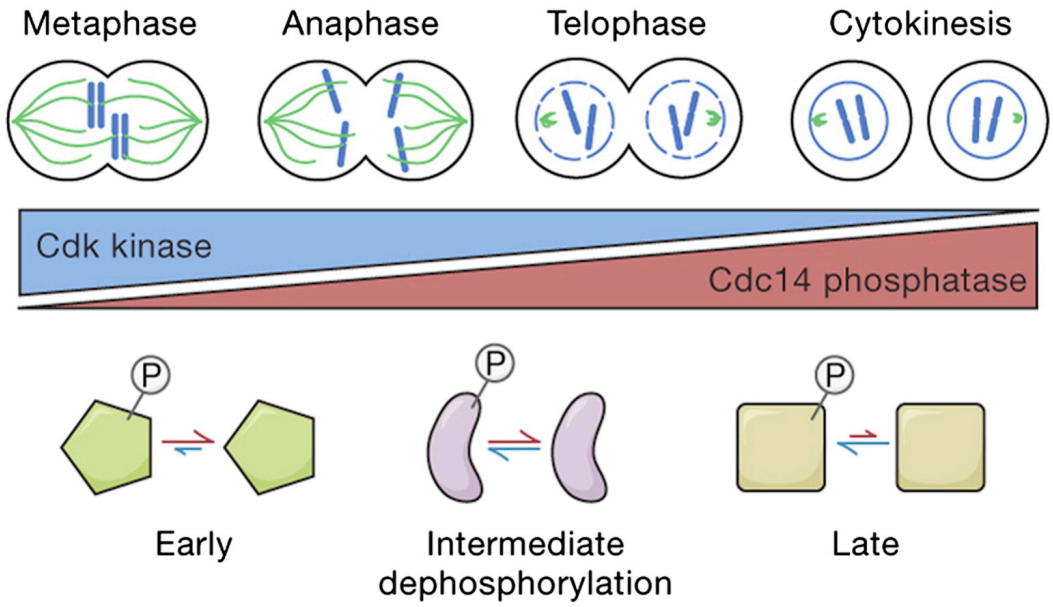



**Figure 1.6 – A quantitative model for Cdk substrate dephosphorylation during mitotic exit**
Cdk substrates are dephosphorylated in an ordered manner depending on the ratio of Cdk to Cdc14 phosphatase activity during mitotic exit. During this time, Cdk activity is declining due to the actions of the APC, and Cdc14 activity is rising due to the successive actions of the FEAR and MEN pathways. From (Bouchoux and Uhlmann, 2011).

### 1.6.2 A quantitative role for phosphatases in interphase

Going back to the quantitative model for the cell cycle, the evidence from mitotic exit can be applied to what is known so far about mitotic entry, and the ordering of S phase and M phase. Removing Cyclin specificity from the picture, we have a system where qualitatively the same kind of (or very similar) Cdk activity, although in quantitatively different amounts, is responsible for ordered phosphorylation of Cdk substrates during interphase. Two possibilities as to how this ordering is achieved are proposed. On the one hand, it could be that Cdk substrates have a wide range of affinities for Cdk (notwithstanding Cyclin Specificity), with the "best" substrates being phosphorylated in early interphase, with low Cdk activity, and the "worst" being phosphorylated in mitosis, when Cdk activity is highest. However, in a space as small and confined as a cell, where Cdk-Cyclin complexes and substrates are in close proximity (and with μM intracellular cyclin concentrations), given the high efficiency of phosphorylation and the speed of substrate turnover – even taking into account substrate competition – it

can be imagined that the worst substrates would be phosphorylated very shortly after the best substrates (Loog and Morgan, 2005; Ghaemmaghami et al., 2003). It is hard to envision how "mitotic" substrate phosphorylation could thus be delayed until mitosis, or how checkpoints would be given adequate time to act. Cdk has an extremely wide range of catalytic efficiencies for substrates, which do not necessarily relate to the order of *in vivo* substrate phosphorylation during the cell cycle (Loog and Morgan, 2005).

On the other hand, it could be that, as observed in mitotic exit, a balance of kinase/phosphatase activity is responsible for ordered phosphorylation (Fig. 1.7). As such, M-phase Cdk substrates would be good substrates for the phosphatase and bad for the kinase, and G1-phase Cdk substrates the opposite. Phosphatase activity would increase the time-resolution of substrate phosphorylation, and allow a built-in lag, or reaction time, for the cell to take action in the event of perturbations such as DNA damage, or SAC activation. Is there any evidence for to support such a model? What could the identity of such a phosphatase be?

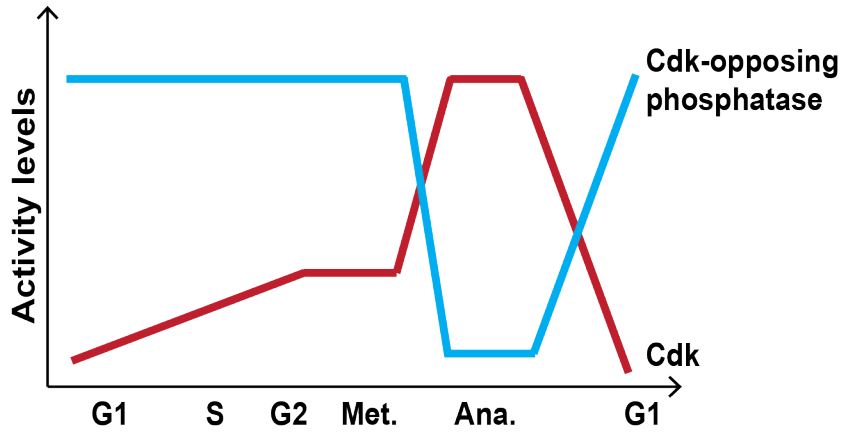



**Figure 1.7 – Cdk-opposing phosphatase activity in the quantitative model for the cell cycle**
Cdk-opposing phosphatase activity may complement Cdk activity in the quantitative model for the cell cycle, allowing for the ordered phosphorylation of Cdk substrates in interphase and ordered dephosphorylation of Cdk substrates during mitotic exit.

## 1.7 Serine/Threonine phosphatases in the *S. cerevisiae* cell cycle

Several Cdk-opposing phosphatases other than Cdc14 are active in the budding yeast cell cycle, and are candidates for opposing Cdk activity in interphase.

### 1.7.1 PP2A plays a variety of roles in the cell cycle

PP2A is probably the best-known Cdk-opposing phosphatase, other than Cdc14, in budding yeast. PP2A is a multi-subunit holoenzyme composed of a scaffold subunit, known as Tpd3, a catalytic subunit, provided interchangeably by the proteins Pph21 or Pph22, and one of three regulatory subunits: Cdc55, Rts1 or Rts3. Regulatory subunits provide PP2A with substrate specificity, with $PP2A^{Cdc55}$ and $PP2A^{Rts1}$ having distinct roles in the cell cycle (Jiang, 2006). The role of Rts3 is unknown. As mentioned previously, $PP2A^{Cdc55}$ maintains Net1 in a hypo-phosphorylated state until the onset of anaphase, thus preventing too early entry into anaphase. This phosphatase also has several other cell cycle roles.

#### *1.7.1.1 $PP2A^{Cdc55}$ promotes mitotic entry*

It has become apparent that $PP2A^{Cdc55}$ plays an important role in controlling the G2/M transition by modulating the levels of inhibitory Y19 phosphorylation on Cdk. In budding yeast, cells lacking Cdc55 are viable (although cold-sensitive) but have altered cell cycle progression, are morphologically abnormal and have a slow growth rate. These bulk of these defects can be rescued by mutation of Y19 on Cdk to a non-phosphorylatable F (Cdc28F19)(Wang and Burke, 1997). Y19 phosphorylation of Cdk1 is in fact carried out by Swe1 (=Wee1), one of the main negative regulators of mitotic entry – $PP2A^{Cdc55}$ has been shown to directly oppose phosphorylation and activation of Swe1 during interphase (Harvey et al., 2011; Lin and Arndt, 1995). In fact, deletion of Swe1 mimics Cdc28F19 in restoring comparatively normal cell cycle progression to Cdc55 mutants. A double deletion strain (*swe1Δ cdc55Δ)* can thus be used to study the

role of Cdc55 in interphase independently of its role in modulating Y19 phosphorylation.

Y19 phosphorylation of Cyclin-Cdk by Swe1 specifically inhibits mitotic Cyclin-Cdk complexes, most strongly reducing the activity of Clb2-Cdk, and also that of Clb3/4-Cdk, but having little effect upon G1 or S phase Cyclin-Cdk complexes. Inhibitory phosphorylation of Y19 (and the timing of its removal) is a major determinant of the timing of mitotic entry. This mechanism of Cdk inhibition is highly conserved; in fact phosphorylation of Y15 on Cdk1 homologs plays a bigger role in mitotic progression in other organisms (including the fission yeast *S. pombe*) than it does in budding yeast. $PP2A^{Cdc55}$ has also been postulated to be a regulator of Mih1 (=Cdc25) by dephosphorylating it, although whether this dephosphorylation is activatory or inhibitory remains unclear (Pal, Paraz and Kellogg, 2008).

The targets and actions of the homologs of $PP2A^{Cdc55}$ (PP2A-B55) in mitotic entry are conserved, however in most other organisms dephosphorylation of Wee1 by PP2A-B55 is activatory rather than inhibitory. Further PP2A-B55 is known to inhibit Cdc25. Therefore PP2A opposes, rather than promotes, mitotic entry in other organisms – the phosphatase is acting upon the same targets, however with opposite effects (Tuck, Zhang, Potapova and Malumbres, 2013; Kinoshita et al., 1993) (Fig. 1.8).

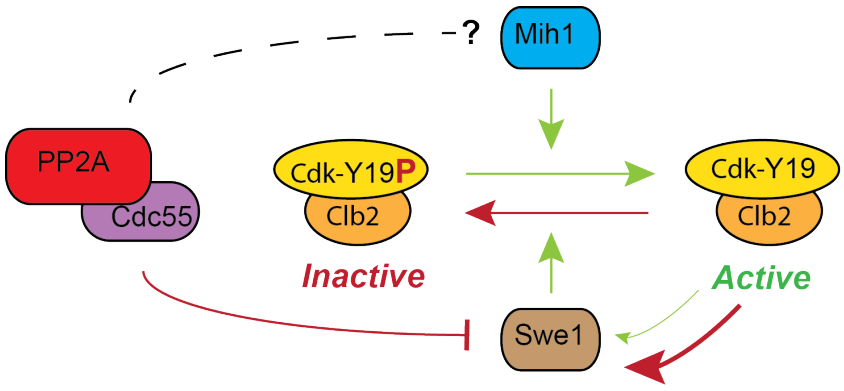



**Figure 1.8 – $PP2A^{Cdc55}$ inhibits Swe1 at the G2/M transition in budding yeast**
$PP2A^{Cdc55}$ plays a role in mitotic entry through the regulation of the phosphorylation state of Cdk-Y19. $PP2A^{Cdc55}$ dephosphorylates and inhibits Swe1, allowing the generation of a low level of Cdk activity in early mitosis.

By inhibiting Swe1 phosphorylation, $PP2A^{Cdc55}$ is a component of the quantitative, "Cdk threshold", model for mitotic progression (Oikonomou and Cross, 2011). It is required for the generation of a stable and low-level plateau of Clb2-Cdc28 activity in early mitosis. In fact, the activating phosphorylation events on Swe1 are themselves carried out by Cdc28-Clb2, an example of the kinase inhibiting its own activation, generating a negative feedback loop (Fig. 1.8). In order to generate a dynamic steady state in which a stable low level of Cdc28-Clb2 activity can be maintained, despite the presence of its inhibitor Swe1, and with rising levels of Clb2, $PP2A^{Cdc55}$ opposes Cdk phosphorylation of Swe1. When Clb2 levels reach a certain threshold, Cdk activity can overcome the inhibition from Swe1, and $PP2A^{Cdc55}$ becomes inhibited by the actions of the FEAR pathway, causing further phosphorylation of Swe1, with hyperphosphorylation being inhibitory, rather than activating, for the protein (Harvey et al., 2011). This results in a dramatic switch from a stable steady state of low Cdk activity to a stable steady state of high Cdk activity, making the transition between these two states irreversible and abrupt. This is another example of a kinase/phosphatase ratio influencing the timing of cell cycle events in a quantitative manner. Interestingly, the Cdk threshold model in mammalian systems also works through Wee1, with initial Wee1 phosphorylation activating the protein, and further phosphorylation inhibiting it, depending on levels of Cdk activity (Deibler and Kirschner, 2010).

#### *1.7.1.2 $PP2A^{Cdc55}$ inhibits anaphase onset*

In a normal cell cycle, Swe1 is degraded shortly after mitotic entry, in order for full Cdk activation to occur. However, when the actin cytoskeleton is perturbed, Swe1 is stabilised and Cdk activity remains inhibited in response to a morphological checkpoint (Lew and Reed, 1995; Sia, 1998). In this case, the cell cycle is arrested in early metaphase, after spindle formation, with high securin levels and no sister chromatid separation. Given the function of $PP2A^{Cdc55}$ in inhibiting Swe1, it seems likely that $PP2A^{Cdc55}$ would be needed for exiting this morphogenesis checkpoint by promoting the downregulation of Swe1. However, it was found that this is not the case. Indeed, the opposite is true; $PP2A^{Cdc55}$ is required for maintenance of the morphogenesis checkpoint. Inactivation of $PP2A^{Cdc55}$ is sufficient to promote sister

chromatid separation despite the presence of an active checkpoint. In fact, cells lacking $PP2A^{Cdc55}$ are also defective in maintenance of the spindle checkpoint, which has been put down to early Cdc14 release in these cells. However, no premature Cdc14 release was seen upon bypass of the morphogenetic checkpoint (Chiroli, Rossio, Lucchini and Piatti, 2007).

This points to $PP2A^{Cdc55}$ as a more general inhibitor of anaphase onset. It has been suggested that $PP2A^{Cdc55}$ inhibits Separase before activation of the APC, under conditions where the sister chromatids are not under tension, to prevent premature chromosome segregation. This adds yet another level of control to the metaphase-anaphase transition (Clift, Bizzari and Marston, 2009).

#### *1.7.1.3 $PP2A^{Rts1}$ is required for the G1/S transition and regulation of cell size*

Less is known about the roles of PP2A in complex with its other regulatory subunit, Rts1. However, it has been established that $PP2A^{Rts1}$ activity promotes the transcription of the G1 cyclins Cln1 and Cln2, possibly through regulation of Swi6. Rts1 deletion is lethal when combined with deletion of Cln1 and Cln2, and alone causes slow growth, a delay in bud emergence and low levels of G1 cyclin transcripts. *rts1Δ* cells are abnormally large, and are unable to alter cell size in response to nutrient availability, indicating that $PP2A^{Rts1}$ is required for nutrient modulation of cell size (Artiles, Anastasia, McCusker and Kellogg, 2009). It is possible that this phosphatase is also required for nutrient modulation of cell size in mitosis via regulation of Swe1.

### 1.7.2 PP1 is essential for cell cycle progression

The final serine/threonine phosphatase in budding yeast – PP1 – is a holoenzyme made up of a core catalytic subunit, the protein Glc7, and a regulatory subunit, of which there are more than 30 (reviewed in (Cannon, 2010)). Glc7 was first identified as being important for glycogen metabolism, but PP1 has since been shown to have a large variety of roles in *S. cerevisiae*. Notably, PP1 is essential for cell cycle progression, with PP1 depletion causing a metaphase arrest (Stark, 1996).

#### *1.7.2.1 PP1 promotes correct kinetochore-microtubule interactions*

Kinetochores need to be properly attached to microtubules for correct chromosome biorientation and chromosome segregation. As such, PP1 antagonises Ipl1 (Aurora) phosphorylation events at the kinetochore, with absence of PP1 causing activation of the spindle checkpoint and halting mitotic progression (Bloecher and Tatchell, 1999). PP1 is targeted to the kinetochore through an interaction with its regulatory subunit and target protein Fin1, where it dephosphorylates Ndc10 and Dam1, promoting mitotic spindle attachment to the kinetochore (Akiyoshi, Nelson, Ranish and Biggins, 2009; Cheeseman et al., 2002; Sassoon et al., 1999).

#### *1.7.2.2 PP1 reverses cell cycle checkpoints*

Not only does PP1 play a role in promoting correct spindle-microtubule attachments, and thus the satisfaction of the spindle assembly checkpoint, but it is also thought to directly silence this checkpoint. Glc7 overexpression allows progression into anaphase even when the spindle checkpoint is defective. Further, Glc7 is required for exit from a transient spindle checkpoint arrest – in the absence of Glc7, cells were unable to escape from a spindle checkpoint arrest caused by addition of the spindle depolymerising drug nocodazole, even after removal of the drug. It is thought that PP1 reverses both Ipl1 and Mps1 (major spindle checkpoint kinases) phosphorylation to actively promote silencing of this checkpoint (Pinsky, Nelson and Biggins, 2009). PP1 is also involved in recovery from inhibition of DNA replication (and activation of the DNA damage checkpoint) (Bazzi et al., 2010).

From looking at the role of Cdk-opposing phosphatases in S. cerevisiae, it seems that any or none of these three phosphatases could have role in directly opposing Cdk phosphorylation in interphase. What does the evidence from other organisms tell us?

## 1.8 Serine/Threonine phosphatases in other organisms

### 1.8.1 The functions of Cdc14 are highly divergent in different organisms

The Cdc14 family of phosphatases is highly conserved, with orthologs having been identified in a wide range of organisms. Cdc14 orthologs have a bipartite structure, with a conserved N-terminal core region, providing catalytic activity, and a non-conserved C-terminal region of variable length. The crystal structure of the N-terminal region of human Cdc14B has revealed two structurally similar domains, arranged in tandem, using which it is possible to create a homology model of budding yeast Cdc14 (Gray, Good, Tonks and Barford, 2003; Mocciaro and Schiebel, 2010) (Fig 1.9). Despite the structural similarities between orthologs, budding yeast Cdc14 (ScCdc14) is the only organism in which Cdc14 activity is a confirmed requirement for mitotic exit.

#### *1.8.1.1 Roles of Clp1, the S. pombe Cdc14 ortholog*

In *S.pombe*, Clp1, the Cdc14 ortholog, behaves in a manner similar to ScCdc14 during interphase, when it is sequestered in the nucleolus. However, it is released at the G2/M transition rather than in anaphase, release that is not dependent on the FEAR pathway (Fig 1.10).. During mitosis, Clp1 dephosphorylates a number of Cdk substrates and regulates spindle stability and chromosome biorientation. Its main role, however, is in cytokinesis, where it regulates the formation of the septum, although it is not essential (Fig 1.10). Its subcellular localisation, and concomitantly, its role in cytokinesis, is regulated by the SIN pathway (homologous to the MEN pathway in budding yeast)(Trautmann et al., 2001; Buttrick et al., 2011).

#### *1.8.1.2 Roles of metazoan Cdc14 orthologs*

In metazoans, the roles of Cdc14 are even more divergent, although relatively little is known about the function of Cdc14 in these organisms. In both *C. elegans* and *X. leavis*, Cdc14 may be important for the completion of cytokinesis. In *G. gallus*, on the other hand, which has two Cdc14 isoforms (A and B), no mitotic role for Cdc14 has yet been

found, although both isoforms might be involved in DNA repair pathways (Fig. 1.10) (Mocciaro and Schiebel, 2010).

In mammalian cells, Cdc14 has three isoforms, A, B and C. Despite being the focus for many studies, the molecular functions of the Cdc14 orthologs in mammalian cells, and whether they have a role in cell cycle progression and mitotic exit, remain unclear. siRNAi against Cdc14A has revealed a role in the regulation of the centrosome cycle, with cells consequently having defects in centrosome separation as well as in mitosis and cytokinesis. Depending on study and experiments used (RNAi depletion/overexpression), human Cdc14B has been suggested to have a role in mitotic spindle assembly, DNA damage checkpoint inactivation, centriole duplication and mitotic exit (Fig. 1.10). However, using gene knockout studies in human cell lines, it has been demonstrated that neither Cdc14A or B are essential for cell viability, and indeed no defects in mitotic progression or centrosome duplication were found in either case. Thus the function of Cdc14 in mammalian systems remains controversial.

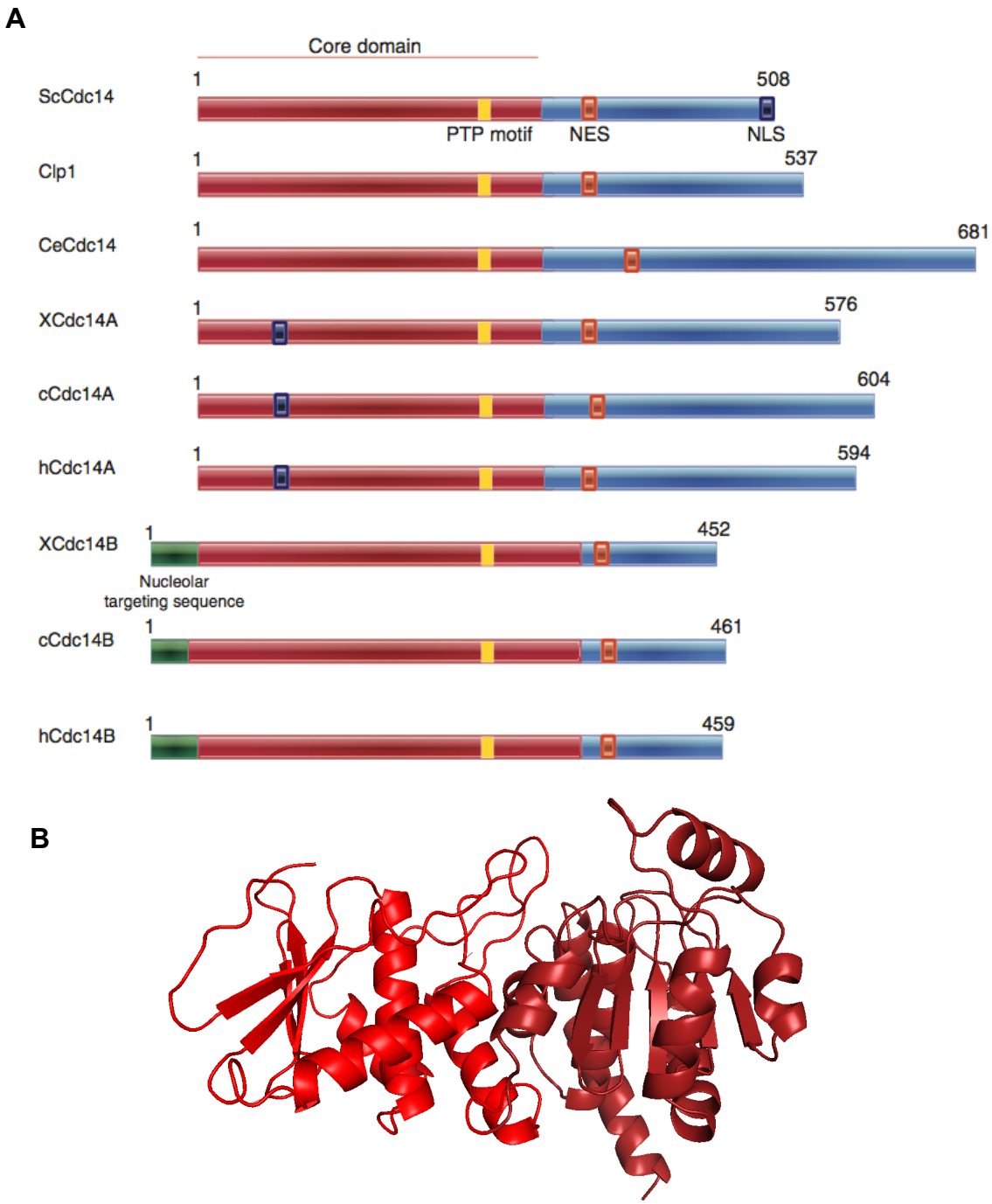



**Figure 1.9 – The structure of Cdc14 orthologs is conserved**
**A.** Cdc14 orthologs have a conserved N-terminal core (red) and a variable C terminal domain (blue). The conserved PTP motif (yellow) forms the catalytic site. *S.cerevisiae* (ScCdc14) *S. pombe* (Clp1), *C. elegans* (CeCdc14), *X. Leavis* (XCdc14A), *G. gallus* (cCdc14A/B) and human (hCdc14A/B) are aligned. Adapted from (Mocciaro and Schiebel, 2010) **B.** Homology model of ScCdc14 conserved domain, based on the structure of the human Cdc14B N-terminal core. In bright red is Domain A, and in dark red Domain B, which provides catalytic activity and contains the PTP motif. Model was made using PyMOL (pymol.org).

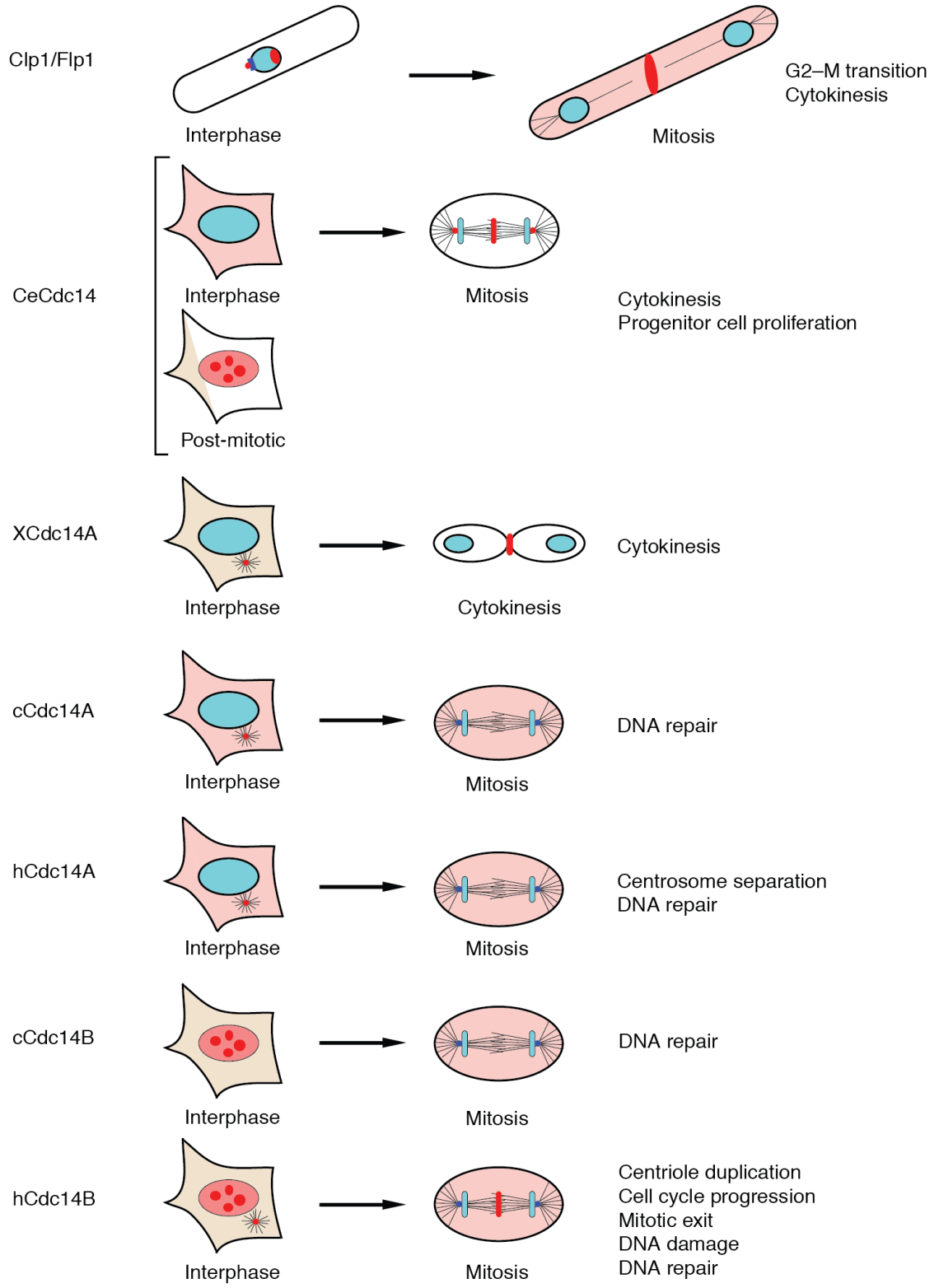



**Figure 1.10 – Cdc14 orthologs have divergent functions**
The roles of *S.pombe* (Clp1), *C. elegans* (CeCdc14), *X. Leavis* (XCdc14A), *G. gallus* (cCdc14A/B) and human (hCdc14A/B) are shown. Cdc14 localisation during interphase and mitosis is depicted in shades of red. Adapted from (Mocciaro and Schiebel, 2010).

### 1.8.2 Mitotic exit requires phosphatase activity in all eukaryotes

Despite the fact that Cdc14 activity is not a requirement for mitotic exit in other organisms, it is likely that a quantitative mechanism for ordered substrate dephosphorylation during mitotic exit is also applicable in eukaryotes other than budding yeast. Indeed, Cdk substrate dephosphorylation seems to be a requirement for mitotic exit, as persistent Cdk activity prevents return to G1 in organisms as diverse as *S. pombe*, *Xenopus* and *Drosophila*, and protein phosphatase activity is a general requirement for progression through mitosis (Parry and O'Farrell, 2001; Murray, Solomon and Kirschner, 1989; Yamano, Gannon and Hunt, 1996; Chen et al., 2007).

In mammalian cells, application of a variety of Cdk inhibitors (including RO-3306, GCP-74514A, flavopiridol, BMI-1026, roscovitine or purvalanol A) has been shown to induce mitotic exit – although not always with very high efficiency – presumably due to the ensuing dramatic drop in Cdk activity (Skoufias et al., 2007; Potapova et al., 2006; Manchado et al., 2010; Niiya et al., 2005). However, in one study, where roscovitine was applied to cells in combination with the drug MG132 (inhibiting proteasomal degradation), mitotic exit no longer occurred, indicating that this drop in Cdk activity is not sufficient to cause a return to G1 (Skoufias et al., 2007). Similarly, adding inhibitors of PP1/PP2A phosphatases (Okadaic acid – OA) together with roscovitine blocks mitotic exit from occurring, with cells arresting in mitosis with condensed chromosomes and high levels of Cdk phosphorylation (Skoufias et al., 2007; Manchado et al., 2010). Hyper-activation of PP2A, along with the application of roscovitine, leads to a semblance of mitotic exit in Mouse Embryonic Fibroblasts (MEFs) lacking Cdc20 and Securin, with Cdk substrates being dephosphorylated and the DNA decondensed, exit prevented by addition of OA. These double mutant cells are usually arrested in metaphase, an arrest that cannot be rescued by the application of roscovitine alone. Hyper-activation of PP1 also leads to partial dephosphorylation of Cdk substrates in Cdc20-null cells in the presence of roscovitine (Manchado et al., 2010).

Together, these results indicate that one or several phosphatases likely need to be activated after destruction of Cyclin B by the APC for mitotic exit to occur, as is the

case in budding yeast, where Cdc14 is activated by $APC^{Cdc20}$ degradation of Securin. Securin itself as the necessary APC target was ruled out in these studies, as was Separase (Skoufias et al., 2007; Manchado et al., 2010).

It has also been shown that depletion of PP2A-B55δ in Xenopus egg extracts in interphase prevented them from exiting mitosis (Mochida, Ikeo, Gannon and Hunt, 2009). Studies aiming to elucidate the identity of the phosphatase necessary for mitotic exit and cytokinesis in mammalian systems have identified PP1 (Wu et al., 2009; Manchado et al., 2010), the Ca(2+)- and calmodulin-dependent phosphatase, Calcineurin (CaN)(Chircop et al., 2010), Fcp1 (Visconti, Palazzo, Monica and Grieco, 2012), PP2A (Schmitz et al., 2010; Garriga et al., 2004; Manchado et al., 2010), as well as Cdc14B (Tumurbaatar et al., 2011). Pocket protein dephosphorylation (the pocket proteins being pRB, p107 and p130) during mitosis has indeed been shown to depend, in a quantitative manner, on the ratio of Cdk/PP2A (or PP2A-like) activity (Garriga et al., 2004).

It seems highly likely that a range of phosphatases cooperate to dephosphorylate Cdk targets and bring about mitotic exit in higher eukaryotes, varying between organisms or even between cell types or developmental stages within the same organism. That this occurs in a quantitative manner is also probable. For instance, it has been established that the activity levels of PP2A-B55δ are stringently regulated by the actions of the mitotic kinase Greatwall, ensuring that the correct balance of kinase to phosphatase activity (high kinase, low phosphatase) is maintained for mitotic phosphorylation to occur. Indeed, Greatwall is required for maintenance of mitosis and its depletion causes mitotic exit to occur in *X. leavis* egg extracts, indicating that the ratio of kinase/phosphatase is the key determinant for mitotic exit (Vigneron et al., 2009).

### 1.8.3 A role for PP2A in interphase

It is also likely that one or several of these phosphatases should play a role in opposing Cdk phosphorylation events in interphase. In the study by Garriga *et al,* it was demonstrated that pocket proteins became abruptly dephosphorylated upon Cdk

inhibition, whatever cell cycle stage this inhibition was carried out at, strongly indicating that Cdk phosphorylation of these proteins is being opposed throughout the cell cycle, even in interphase (Garriga et al., 2004).

Indeed, in *X. leavis,* PP2A-B55δ has a role in interphase as well as mitotic exit. Immunodepletion of this phosphatase in interphase egg extracts causes early entry into mitosis, and its inhibition triggers mitotic entry even with very low levels of Cyclin-Cdk activity (Krasinska et al., 2011; Mochida et al., 2009). Indeed, PP2A downregulation (by Greatwall) is necessary for mitotic entry as well as mitotic exit in this organism (Castilho et al., 2009; Vigneron et al., 2009). Mathematical modelling indicates that PP2A activity is necessary for S phase and mitosis to be mutually exclusive states, and to promote dynamic transitions between these two states (Krasinska et al., 2011).

Further, increasing the levels of recombinant PP2A-B55δ in egg extracts leads to a dose-dependent delay in mitotic progression (Mochida et al., 2009). Therefore the timing of interphase events, and the ordering of S phase and mitosis in *X. leavis*, is determined by a balance between opposing kinase and phosphatase activities in a quantitative manner (Fisher, Krasinska, Coudreuse and Novak, 2012). It is possible that this might be due to an effect upon the Wee1-Cdc25 loop, as noted in budding yeast. However, it is also possible that this is more direct – high PP2A levels in interphase acting as a "buffer" to prevent early mitotic entry through phosphorylation of Cdk1 targets.

## 1.9 Aims and outline of this thesis

Our knowledge of cell cycle regulation by phosphatases has been increasing over the past decades. However, much remains to be elucidated, both in the well-characterized process that is mitotic exit, and regarding the role of Cdk-opposing phosphatases in other cell cycle stages. In this thesis, I hope to present new insights into both of these aspects of cell cycle regulation, using *S. cerevisiae* as a model organism. In particular, I have focused on answering two questions.

### 1.9.1 What are the Cdc14 targets necessary for rDNA condensation and or/segregation in early anaphase?

As discussed extensively in this chapter, the role of Cdc14 in budding yeast mitotic exit, and the regulation of the activity of this phosphatase, are relatively well understood. In particular, it is well established that Cdc14 functions to promote the condensation and/or segregation of the rDNA repeats in early to mid-anaphase. However, the exact targets upon which Cdc14 is acting to promote these processes remain unclear. Therefore, my initial aim was to identify whether any such targets exist, and if so, how Cdc14 is regulating them in order to bring about these morphological changes within the nucleolus. Ultimately, I was unsuccessful in identifying any such targets, however my research led me to uncover a novel mechanism for the regulation of Cdc14 activity during anaphase. My results shall be presented and discussed in Chapter 3.

### 1.9.2 Do Cdk-opposing phosphatases play a role in determining the timing of progression through interphase and entry into mitosis?

In a second time, and after having focused on furthering our understanding of the role of mitotic phosphatases in mitotic exit, I next concentrated on the regulation of interphase by these phosphatases. As such, evidence from budding yeast mitotic exit and mitotic progression, as well as from *X. leavis* egg extracts, indicates that Cdk-opposing phosphatases may be a necessary part of a quantitative model for the cell cycle. In this model, the ratio of kinase/phosphatase activity determines the ordered phosphorylation and dephosphorylation of mitotic phosphoproteins. This has been

demonstrated to be the case in budding yeast mitotic exit; whether it is also important for the ordering of S phase events and mitotic entry is what I have aimed to determine. I have seen that the phosphatase $PP2A^{Cdc55}$ may play a role in controlling the timing of Cdk substrate phosphorylation during interphase, which shall be the topic of Chapter 4.

Finally, in Chapter 5, I shall discuss, more generally, the implications of my findings in deepening our current understanding of cell cycle regulation.

# Chapter 2. Materials & Methods

## 2.1 Yeast techniques

### 2.1.1 Yeast strains and growth conditions

All budding yeast strains used in this study were of W303 background (Table 2.2) apart from strains 2369, 4839 and 4848, which were of the S288C background. Cells were grown in YP (Yeast Peptone) supplemented with 2% w/v glucose (YPD – Yeast Peptone Dextrose) or 2% w/v raffinose/galactose (YP-Raff/Gal) (Table 2.1). For SILAC experiments, cells were grown in synthetic YNB media (Table 2.2) and 60 μg/ml of each of the following amino acids: tyrosine, uracil, tryptophan, leucine, adenine, histidine, isoleucine and phenylanine, and 50 μg/ml threonine, as well as 100 μg/ml of either heavy (13C), or light (12C), lysine or arginine, supplemented with 2% w/v glucose. For the selection of transformants, YNB agar plates were used lacking the auxotrophic amino acid used for selection. For selection based on Kanamycin resistance YPD agar plates were supplemented with the kanamycin derivative Geneticin G418 (50 μg/ml). Cells were sporulated on sporulation media. Strains harboring conditional Clb2-fusion cassettes were grown on medium lacking uracil to maintain the selectable marker and to prevent spontaneous recombination. Marker loop-out was then induced by β-estradiol-dependent activation of Cre recombinase fused to an estradiol binding domain (Cre-EBD78), by addition of 1 mM β-estradiol to the growth medium.

| Medium name/ Abbreviation | Composition |
|---|---|
| YP | Yeast Peptone – 1.1% w/v yeast extract, 2.2% w/v bacto-peptone and 0.0055% w/v adenine-HC |
| YNB | Yeast Nitrogen Base – 0.8% w/v yeast nitrogen base |
| YP agar | 1.1% w/v yeast extract, 2.2% w/v bacto-peptone and 0.0055% w/v adenine-HC, 2% w/v agar |
| YNB agar | 0.8% w/v yeast nitrogen base, 2% w/v agar |
| Sporulation media | 100 mM $CH_3COONa$, 20 mM NaCl, 25 mM KCl, 1.5mM $MgSO_4$ and 1.5% w/v agar |

**Table 2.1 – Media composition**

### 2.1.2 Strain List

| Strain number - Lab collection/personal collection (MG..) | Description | Mating type |
|---|---|---|
| **141** | W303 | a |
| **4652** | Nur1-PK3::TRP | a |
| **4653** | Nur1-PK3::TRP *cdc14-1* | a |
| **4565** | trp1::TRP1-CRE-EBD78 | a |
| **4654** | Nur1-Clb2::URA trp1::TRP1-CRE-EBD78 | a |
| **4655** | Nur1-Clb2ΔCDK::URA trp1::TRP1-CRE-EBD78 | a |
| **4656** | W303R (RDN1::ADE2 Rad5) | a |
| **4657** | W303R trp1::TRP1-CRE-EBD78 | a |
| **4658** | W303R trp1::TRP1-CRE-EBD78 Nur1-Clb2::URA | a |
| **4659** | W303R trp1::TRP1-CRE-EBD78 Nur1-Clb2ΔCDK::URA | a |
| **4660** | Nur1-Clb2::URA Net1-YFP::HIS trp1::TRP1-CRE-EBD78 | a |
| **4661** | Nur1-Clb2ΔCDK::URA Net1-YFP::HIS trp1::TRP1-CRE-EBD78 | a |
| **4662** | Nur1-Clb2::URA Cdc14-GFP::LEU trp1::TRP1-CRE-EBD78 | a |
| **4663** | Nur1-Clb2ΔCDK::URA Cdc14-GFP::LEU trp1::TRP1-CRE-EBD78 | a |
| **4664** | *nur1Δ*::LEU | a |
| **4665** | *nur1(9A)*::LEU | a |
| **4666** | *nur1Δ*::LEU Cdc14-GFP::TRP | a |
| **4667** | *nur1(9A)*::LEU Cdc14-GFP::TRP | a |
| **4668** | *nur1Δ*::LEU Cdc14-GFP::TRP *Δspo12*::HIS | a |
| **4669** | *nur1(9A)*::LEU Cdc14-GFP::TRP *Δspo12*::HIS | a |
| **4670** | *spo12Δ*::HIS Cdc14-GFP::TRP | a |
| **4671** | Cdc14-GFP::TRP | a |
| **4672** | Nur1(9A)-Clb2::URA trp1::TRP1-CRE-EBD78 | a |
| **4673** | Nur1(9A)Clb2ΔCDK::URA trp1::TRP1-CRE-EBD78 | a |
| **4675** | Nur1-Clb2::URA trp1::TRP1-CRE-EBD78 *TAB6-1* Cdc14-PK3::LEU | a |
| **4676** | Nur1-Clb2ΔCDK::URA trp1::TRP1-CRE-EBD78 *TAB6-1* Cdc14-PK3::LEU | a |
| **844** | *dbf2-2* | α |

| | | |
|---|---|---|
| **145** | *cdc15-2* | a |
| **4677** | *nur1Δ*::LEU *dbf2-2* | α |
| **4678** | *nur1Δ*::LEU *cdc15-2* | a |
| **4679** | Net-myc9::TRP Cdc14-HA6::HIS Nur1-PK3::LEU | a |
| **4873** | *swe1Δ*::HIS | a |
| **4874** | *swe1Δ*:HIS *cdc55Δ*::LEU | a |
| **MG21** | *swe1Δ*::HIS Ndd1-HA3::TRP | a |
| **MG14** | *swe1Δ*:HIS *cdc55Δ*::LEU Ndd1-HA3::TRP | a |
| **MG73** | *swe1Δ*::HIS Sli15-HA3::TRP | a |
| **MG74** | *swe1Δ*:HIS *cdc55Δ*::LEU Sli15-HA3::TRP | a |
| **MG124** | *swe1Δ*::HIS Acm1-HA3::TRP | a |
| **MG125** | *swe1Δ*:HIS *cdc55Δ*::LEU Acm1-HA3::TRP | a |
| **2369** | *arg4Δ*:KANMX4 *lys1Δ*::KANMX4 | a |
| **4839** | *arg4Δ*:KANMX4 *lys1Δ*::KANMX4 *swe1Δ*::HIS | a |
| **4848** | *arg4Δ*:KANMX4 *lys1Δ*::KANMX4 *swe1Δ*:HIS *cdc55Δ*::LEUs | a |
| **MG128** | *cdc28-as1 swe1Δ*::HIS | a |
| **MG129** | *cdc28-as1 swe1Δ*::HIS *cdc55Δ*::LEU | a |
| **1262** | *cdc14-1* | a |

**Table 2.2 – Strain list**

### 2.1.3 Cell synchronization

Budding yeast cells of the "a" mating type were arrested in G1 with the mating pheromone α-factor. To arrest cells, early log phase cultures (OD600 = 0.15) were treated with α-factor (provided by Peptide Synthesis Laboratory, Crick Institute Lincoln's Inn Fields Laboratory) at a concentration of 2.5 μg/ml. The same amount of α-factor was added to cultures every 50 minutes during two hours and 30 minutes. Arrest was determined cytologically by the appearance of a pear-shaped "schmoos" and by FACS (Fluorescence-activated cell sorting) analysis of DNA content. G1 arrested cells were released by filtration with media devoid of α-factor and sugar. For filtration, cells were collected on a membrane filter using filtration apparatuses of different sizes depending on culture volume. Cells were washed with YP or YNB (>6 times culture volume) and released into YP or YNB media supplemented with the appropriate source of sugar and appropriate amino acids. For temperature sensitive strains cells were shifted to restrictive temperature at the time of release. For re-arrest in the following

G1, 3 times the initial concentration of α-factor was used if cells were released at 25°C, and 6 times the initial concentration of α-factor for cells released at temperatures > 35.5°C.

### 2.1.4 Yeast transformation

Yeast transformation was performed using purified PCR products for epitope-tagging or gene disruption, or with linearised plasmid DNA for integration of promoter-gene cassettes or exchange of endogenous promoters of essential genes.

About 50 ml of a mid-log-phase culture (OD 0.2-0.5) was pelleted at 3,000 rpm for 5 minutes. The cell pellet was washed with 1 ml of distilled water and then with 1 ml TEL (10 mM Tris/HCl pH 7.5, 100 mM EDTA and 100 mM Lithium acetate) before being resuspended in 100 µl TEL. 1 µg of either linearised vector DNA or PCR product was mixed with 2 µl of 10 mg/ml single stranded salmon sperm carrier DNA and 300 µl TELP (TEL plus 40% PEG 3350 or 4000). The cell suspension was added to this mix followed by a short vortex. After incubation at 25°C for 2-4 hours, cells were heat shocked at 42°C for 15 minutes. They were then pelleted at 6,000 rpm for 2 minutes, washed in 1 ml sorbitol and plated on selective media. Transformants were checked for the correct integration of the PCR cassette by PCR or Western blot analysis.

### 2.1.5 Yeast mating and tetrad dissection

Mating was induced by incubation of opposite mating type yeast strains on YPD plates at 25°C for 12 hours. Diploids were selected on appropriate selective media and grown on YPD for 24 hours. To induce sporulation, diploids were streaked on a sporulation plate for 2-3 days. Spores were then resuspended in 1 M sorbitol and treated with Lyticase (Sigma-Aldrich) at 30°C for 10 minutes to break the asci. The four released spores from each ascus were dissected using a Singer-MSM micromanipulator and incubated at 25°C until colony formation.

## 2.2 Biochemistry

### 2.2.1 Preparation of protein extracts

Protein extracts were prepared by precipitation with trichloroacetic acid (TCA), washed with 1 M Tris-base and resuspended in 100 µl 2xSDS buffer (100 mM Tris-HCl pH 6.8, 200 mM DTT, 4% SDS, 0.1% Bromophenol blue, 20% glycerol). 200 µl of 0.5 mm glass beads (Biospec Products, Inc) were added and cells were lysed using a FastPrep at 5.5 m/s for 1 minute. Extracts were then spun down to separate them from the glass beads. Collected supernatant was boiled at 95°C for 2 minutes and cleared by briefly centrifuging at 13,000 rpm.

### 2.2.2 SDS-polyacrylamide gel electrophoresis (Page) and Western blotting

Protein samples were resolved on acrylamide/bis-acrylamide gels (37.5:1, 30% solution), with 375 mM Tris-HCl pH 8.8 and 0.1% SDS. Concentrations of acrylamide between 7% and 12% were used, depending on protein size. Proteins were migrated at 50 mA using SDS-PAGE running buffer (25 mM Tris, 250 mM glycine and 0.1% SDS) in electrophoresis tanks from CBS scientific, CA. For Phos-Tag acrylamide gels, 100 µm MnCl2 and 50 µM Phos-tag (from 5 mM stock in methanol, Wako Chemicals), were added. A broad range pre-stained protein marker (New England Biolabs) was added to determine protein migration, (although not to Phos-Tag acrylamide gels).

Separated proteins were transferred onto pre-equilibrated nitrocellulose membranes using a wet-transfer tank (Biorad). Transfer buffer contained 3.03 g/l Tris base, 14.1 g/l glycine, 0.05% SDS and 20% v/v methanol, and was carried out either at 250 mA for 12 hours or 400 mA for 2 hours. In the case of Phos-Tag acrylamide gels, gels were incubated in transfer buffer with 10 mM EDTA for 2 x 10 minutes and in transfer buffer alone for 1 x 10 minutes before transfer to eliminate the $Mn^{2+}$ ions from the gel.

The membrane was blocked with a 4% skimmed milk solution (Marvel) in PBST (Phosphate Buffered Saline Tween) (170 mM NaCl, 3 mM KCl, 10 mM Na2HPO4, 2

mMKH2PO4, 0.01% tween 20) for 30 minutes - 1 hour at room temperature. Membranes were then incubated with primary antibodies diluted in PBST containing 4% milk for 1-2 hours at room temperature or at 4°C overnight. Antibodies used for Western detection are detailed in Table 2.2. : α -Clb2 (Santa Cruz, sc9071), α-Orc6 (clone SB49); α-Sic1 (Santa Cruz, sc50441), α-Tub1 (clone YOL1/34, AbD Serotec), α-Tat1 (Sigma-Aldrich) α-HA (clone 12CA5), α-myc (clone 9E10), α-Pk (clone SV5-Pk1, AbD Serotec). Membranes were washed for 3 x 10 minutes with PBST before being incubated with α-mouse, α-rabbit or α-rat horseradish peroxidase (HRP) coupled secondary antibodies in PBST containing 4% milk for 30 minutes – 2 hours at room temperature. Membranes were washed a further three times before adding ECL (Amersham) according to the manufacturer's instructions, and then were either developed using a developer or photographed using an ImageQuant las 4000 (GE Healthcare).

### 2.2.3 Immunoprecipitation

For immunoprecipitation, cell extracts were prepared in EBXG buffer (50 mM HEPES pH 8.0, 100 mM KCl, 2.5 mM $MgCl_2$, 10% glycerol, 0.25% Triton X-100, 1 mM DTT, cOmplete protease inhibitor tablets - Roche) using glass bead breakage in a Multi Bead Shocker (Yasui Kikai) at 2°C. Extracts were cleared by centrifugation at 14800 rpm for 30 minutes at 4°C, pre-cleared using Protein A Dynabeads for 2 hours, incubated with antibody for 1 hour, and finally adsorbed to Protein A Dynabeads for 2 hours. Beads were washed in EBXG and elution was carried out in SDS-PAGE loading buffer at 65°C for 20 minutes.

### 2.2.4 Phosphatase assay

For the *in vitro* Nur1 dephosphorylation assay, immunoprecipitation was performed as above, then beads were resuspended in phosphatase buffer (New England Biolabs) and 1 μg λ phosphatase (New England Biolabs), or 8 μg purified recombinant Cdc14, kindly provided by C. Bouchoux (Bouchoux and Uhlmann, 2011), were added, followed by incubation at 30°C for 30 minutes before the reaction was stopped and proteins eluted by addition of SDS-PAGE loading buffer.

## 2.3 Molecular Biology and DNA manipulation

### 2.3.1 Genomic DNA preparation

Yeast genomic DNA for PCR genotyping was prepared from fresh patches of the strain of interest. Cells was resuspended in 100 µl 200 mM LiOAc 1% SDS solution, and incubated at 70°C for 15 min. After incubation, 300 µL 100% ethanol was added, and DNA was collected by centrifugation at 13000 rpm for 3 min. DNA Pellets were dried for 15 minutes at 30°C and dissolved in 50-100 µL TE. Cell debris was spun down by brief centrifugation (13000 rpm for 1 min), and 1 µL supernatant was used for PCR.

### 2.3.2 Polymerase Chain Reaction (PCR)

PCR reactions were carried out in 25 or 50 µl reactions containing Taq (Qiagen), Expand High Fidelity (Roche), or ClonAmp (ClonTech) polymerases with buffers supplied by the manufacturers, and if necessary 0.2 mM dNTPs as well as 0.5 µM of each primer. All PCRs were performed on a Peltier Thermal Cycler (MJ Research). PCR products were resolved by agarose gel electrophoresis to confirm the size of the fragments.

### 2.3.3 Strain design

#### *2.3.3.1 Epitope tagging*

Epitope tagging of endogenous genes was performed by gene targeting using polymerase chain reaction (PCR) products (Bähler et al., 1998; Knop et al., 1999).

Forward and reverse primers with homology to respectively the 3' end of the gene of interest and the 3'UTR were used, also containing an 18-mer sequence homologous to the vector used for tagging. These were used to amplify the vector by PCR, with the subsequent product containing the epitope tag, a marker, and a 50-mer sequence at each end homologous to the region of marker insertion.

Transformants were subsequently selected on plates by using auxotrophic markers (derived from either *Kluyveromyces lactis* or *S. pombe)* to minimize the chance of integration at the marker locus, or G418 resistance.

To generate the conditional Nur1-Clb2 and Nur1-Clb2ΔCdk fusions, as described in (Kuilman et al., 2015), Clb2 lacking its destruction and KEN-boxes (Wäsch and Cross, 2002), as well as its nuclear localization sequence (Eluère et al., 2007), was fused to Nur1, separated by an unstructured 10-mer GGSGTGGSGT linker. In addition, the Clb2 fusion cassette contained an HA-epitope tag before Clb2, flanked by two LoxP sites, and an HA-epitope tag after Clb2. The Clb2ΔCdk mutant further contained 3 point mutations (K316A, E345, F354A) that prevent it from interacting with the Cdc28 kinase subunit (Bailly et al., 2003). The Clb2 fusion cassette, constructed on a plasmid, was amplified by PCR and targeted to Nur1 using homologous primers.

#### *2.3.3.2 Gene replacement and deletion*

Genes were deleted using PCR generated products (Bähler et al., 1998). A marker gene (LEU2, URA3, HIS3, TRP1) or KanMX6 was amplified with flanking sequences corresponding to the 5' and 3' ends of the relevant genes. Fragments were transformed into a strain lacking the selective marker. Colonies were selected on plates lacking the selective marker.

The *nur1(9A)* mutant was engineered by endogenous gene replacement using an integrative plasmid, based on a synthetic DNA construct (GeneArt, Life Technologies).

#### *2.3.3.3 PCR programme for C-terminal tagging and deletion of yeast proteins*

| Step | Time | Temperature (°C) |
|---|---|---|
| 1 | 2 minutes | 94 |
| 2 | 15 seconds | 94 |
| 3 | 45 seconds | 50-60 depending on primer tm |
| 4 | 1 minute 30 seconds | 72 |
| 5 | Go to step 2 and repeat 5X | - |
| 6 | 15 seconds | 94 |
| 7 | 45 seconds | 55-65 depending on primer tm |
| 8 | 1 minute 30 seconds + 5 seconds/cycle | 72 |
| 9 | Go to step 6 and repeat 25X | - |
| 10 | 7 minutes | 72 |
| 11 | - | 4 |

**Table 2.3 – PCR programme used for gene deletion and epitope tagging**

### 2.3.4 Vector List

| Number | Description |
|---|---|
| 35 | One-step C terminal tagging vector – HA3::K.l TRP |
| 554 | One-step C terminal tagging vector – PK3::K.l TRP |
| 39 | One-step C terminal tagging vector – myc18::K.l URA |
| 32 | Gene deletion vector (pBlueScript Backbone) – K.l TRP |
| 210 | Gene deletion vector (pBlueScript Backbone) – K.l URA |
| 211 | Gene deletion vector (pBlueScript Backbone) – K.l LEU |
| 212 | Gene deletion vector (pBlueScript Backbone) – K.l HIS |
| 1109 | YIplac128-URA3-l30-Clb2 (conditional fusion construct) |
| 1110 | YIplac128-URA3-l30-Clb2ΔCdk (conditional fusion construct) |
| 518 | YIplac211-GAL-Cdc14-Pk3::URA3 |
| MG1 | prS305-Nur1(9A)- LEU2 |

**Table 2.4 – Vector List**

### 2.3.5 Agarose gel electrophoresis

DNA samples were loaded on a 0.8-2% agarose gel (depending on the size of the DNA fragment) in 6x loading buffer (0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol, 30% (v/v) glycerol). Agarose gels were prepared in 1x Tris-acetate EDTA buffer (TAE) (40 mM Tris base pH 7.5, 1 mM EDTA pH 8.0, 0.115% v/v acetic acid), to which GelRed (Biotium, Inc.) was added to a final concentration of 0.5 μg/ml. Electrophoresis was carried out in TAE at 80- 120 V in electrophoresis tanks from Anachem.

## 2.4 Microscopy and Cell Biology

### 2.4.1 Spheroblastation

Cells were fixed in buffer IF1 (100 mM potassium phosphatase pH 6.8, 0.5 mM MgCl2) with 3.7% formaldehyde, washed in buffer IF1 without formaldehyde, then washed again in buffer IF2 (100 mM potassium phosphatase pH 7.4, 0.5 mM MgCl2, 1.2 M Sorbitol). Cells were incubated at 37°C for 30 minutes in spheroblasting buffer (IF2 with 2% lyticase and 2% β-mercaptoethanol), washed once in IF2, and resuspended in IF2 before stored at – 20°C.

### 2.4.2 Immunofluorescence

Indirect immunofluorescence was performed on formaldehyde-fixed, spheroblasted cells, using 15-well slides (MP Biomedicals). Cells were fixed on the slides using Poly-l-lysine, and mounted using ProLong Gold. The antibodies used for immunofluorescence are described in table 2.3The following antibodies were used: α-GFP, (clone TP401, Torrey Pines Biolabs or ab6556, Abcam), α-Tub1 (clone YOL1/ 34, AbD Serotec) and FITC and Cy3-dye labeled secondary antibodies (Sigma and Chemicon, respectively). Cells were counterstained with the DNA binding dyes 4',6-diamidino-2- phenylindole (DAPI), or Hoescht, present in the mounting media ProLong Gold at a concentration of 100 ng/ml. Fluorescent images were acquired using an Axioplan 2 imaging microscope (Zeiss) equipped with a 100x (NA = 1.45) Plan-

Neofluar objective and an ORCA-ER camera (Hamamatsu). Spindle length measurements were carried out in ImageJ.

### 2.4.3 Cell cycle analysis by Flow Cytometry (FACS)

Cells were fixed in ethanol for 2-24 hours at 4°C, RNase treated in 50 mM Tris-HCl pH 7.5 with 0.1 mg/ml RNase A for 2-24 hours at 37°C, and resuspended in propidium-iodide containing buffer (200 mM Tris-HCl pH 7.5, 211 mM NaCl, 78 mM MgCl2, 50µg/ml propidium iodide). Cells were sonicated (Sanyo, Soniprep 150) before being analysed on a FACScan, or a FACSCalibur (Becton Dickinson). DNA content profiles were prepared using FlowJo.

## 2.5 Stable Isotope Labelling In Culture

### 2.5.1 Sample preparation

Cell cultures were grown for > 8 generations in heavy or light media, arrested and released then mixed and collected. 45 ml of cells was added to 5 ml 100 % TCA, and the samples left to precipitate on ice for >30 min. Cells were spun down and the pellets washed with acetone. Pellets were resuspended in beating buffer (8 M urea, 5 mM EDTA, 50 mM ammonium bicarbonate). 0.5 mm glass beads (Biospec Products, Inc) were added and the cells were lysed using glass bead breakage in a Multi Bead Shocker (Yasui Kikai) at 2°C. The supernatant was separated from the beads and spun down at 14800 rpm for 10 min, to remove cell debris. Supernatant was collected and frozen at -80°C.

### 2.5.2 Sample processing for mass spectrometry

From this point onwards, all procedures were carried out by members of the Crick Protein Analysis and Proteomics facility.

Samples were reduced with 1 M dithiothreitol (DTT) for 25 min at 56°C, alkylated with 500 mM iodoacetamide for 30 min at room temperature protected from light and quenched with 1 M dithiothreitol. Samples were then diluted with 50 mM ammonium

bicarbonate to reduce the urea concentration to <2 M prior to an overnight trypsin digestion at 37°C. The peptides were acidified to 0.4 % with trifluoroacetic acid (TFA) and centrifuged at 14000 rpm at 4°C for 30 min. Peptides were then desalted using a $C_{18}$ SepPak Lite, 130 mg bed volume, under vacuum and dried. They were then digested using Lys-C in 10 % acetonitrile, 50 mM ammonium bicarbonate at 37°C for 2 h, followed by trypsin digestion at 37°C overnight. Digested peptides were then again desalted and dried.

Titanium dioxide enrichment was then carried out: dried fractions were re-suspended in 80% acetonitrile, 5% trifluoroacetic acid, 1 M glycolic acid, sonicated for 10 min and added to 5 mg of titanium dioxide beads. The beads were washed using 80% acetonitrile and 1% trifluoroacetic acid followed by 10% acetonitrile and 0.2% trifluoroacetic acid, and dried under centrifugation. Phosphopeptides were then eluted from the beads by adding 1% ammonium hydroxide followed by 5% ammonium hydroxide, and dried by vacuum centrifugation.

Dried phosphopeptides were then re-suspended in 100 µl of 1% trifluoroacetic acid and sonicated for 15 min. A $C_{18}$ membrane was packed into a 200 µl pipette tip and washed using methanol and equilibrated with 1% trifluoroacetic acid. The peptides were then loaded onto the Stage Tip and washed with 1% trifluoroacetic acid followed by elution with 80% acetonitrile, 5% trifluoroacetic acid. The eluted peptides were again dried under vacuum centrifugation.

### 2.5.3 Data acquisition by mass spectrometry

For LC-MS/MS data acquisition analysis, the following was performed: Peptide mixtures were re-suspended in 35 µl 0.1% trifluoroacetic acid, and injected three times (10 µl per injection). Each run consisted of a 3 h gradient elution with one activation method per run: CID, MSA and HCD. An LTQ-Orbitrap Velos was used for data acquisition.

Data processing was performed using the MaxQuant bioinformatics suite as adopted by the Crick Protein Analysis and Proteomics facility, and protein database searching was

performed by the Andromeda search engine using a UniProt database of *S. cerevisiae* proteins amended with common contaminants. Data was analysed using the Perseus software package.

## 2.6 Summary of antibodies used

Antibodies used for western blot (WB), immunoprecipitation (IP) and immunofluorescence (IF), and the concentrations used are detailed in table 2.5.

| Antigen | Provenance and description | Dilution/Amount | | |
|---|---|---|---|---|
| | | WB | IP | IF |
| Clb2 | Santa Cruz, sc9071 (0.2 mg/ml) - Rabbit | 1/500-1/1000 | | |
| Orc6 | In house, clone SB49 (1.1 mg/ml) - Mouse | 1/1000 | | |
| Sic1 | Santa Cruz, sc50441 (0.2 mg/ml) - Rabbit | 1/500 | | |
| Tub1 | Clone YOL1/34, AbD Serotec (1 mg/ml) - Rat | 1/1000 | | 1/200 |
| Tat1 | Sigma-Aldrich (1.5 mg/ml) - Mouse | 1/10000 | | |
| HA | Clone 12CA5, Abcam (1 mg/ml) - Mouse | 1/5000 | | |
| myc | In house, clone 9E10 (1.2 mg/ml) - Mouse | 1/5000 | 5 µg | |
| Pk | Clone SV5-Pk1 AbD Serotec - Mouse | 1/5000 | | |
| GFP | clone TP401, Torrey Pines Biolabs (1 mg/ml) – rabbit | | | 1/100 |
| GFP | Ab6556, abcam - Rabbit | | | 1/100 |
| Mouse IgG | GE Healthcare – HRP-coupled secondary | 1/5000 | | |
| Rabbit IgG | GE Healthcare – HRP-coupled secondary | 1/5000 | | |
| Rat IgG | GE Healthcare – HRP-coupled secondary | 1/5000 | | |
| Rat IgG | Sigma – Cy3-Coupled secondary | | | 1/500 |
| Rabbit IgG | Chemicon – FITC-coupled secondary | | | 1/200 |

**Table 2.5 – Antibody list**

## 2.7 Summary of buffers and solutions used and their compositions

| Buffer name/ Abbreviation | Composition | Section Ref. |
|---|---|---|
| TL | 10 mM Tris/HCl pH 7.5, 100 mM EDTA, 100 mM Lithium acetate | 2.1.3 |
| TELP | TEL plus 40% Poly-ethylene-glycol 3350 or 4000 | 2.1.3 |
| Tris-Base | 1 M Tris-Base (no pH) | 2.2.1 |
| 2X SDS | 100 mM Tris-HCl pH 6.8, 200 mM DTT, 4% SDS, 0.1% Bromophenol Blue, 20% Glycerol | 2.2.1 |
| SDS-Page running buffer | 25 mM Tris, 250 mM Glycine, 0.1% Sds | 2.2.2 |
| Transfer Buffer | 3.03g/l Tris base 14.1 g/l glycine, 0.05%SDS 20% w/v methanol | 2.2.2 |
| PBST | Phosphatase Buffered Saline Tween – 170 mM NaCl, 3 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, 0.01% Tween20 | 2.2.2 |
| EBXG | 50 mM HEPES pH 8.0, 100 mM KCl, 2.5 mM $MgCl_2$, 10% glycerol, 0.25% Triton X-100, 1 mM DTT, cOmplete protease inhibitor tablets - Roche | 2.2.3 |
| DNA extraction | 200 mM LiOAc, 1% SDS | 2.3.1 |
| TE | 10 mM Tris-HCl pH 8, 1 mM EDTA | 2.3.1 |
| 6X DNA loading buffer | 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol, 30% (v/v) glycerol | 2.3.4 |
| TAE | 40 mM Tris base pH 7.5, 1 mM EDTA pH 8.0, 0.115% v/v acetic acid | 2.3.4 |
| IF1 | 100 mM potassium phosphatase pH 6.8, 0.5 mM MgCl2 | 2.4.1 |
| IF2 | 100 mM potassium phosphatase pH 7.4, 0.5 mM MgCl2, 1.2 M Sorbitol | 2.4.1 |
| Spheroblasting buffer | IF2 + 0.2% lyticase and 0.2% β-mercaptoethanol | 2.4.1 |
| 70% EtOH | 70% ethanol in H2O | 2.4.2 |
| RNase buffer | 50 mM Tris-HCl pH 7.5 | 2.4.2 |
| FACS buffer | 200 mM Tris-HCl pH 7.5, 211 mM NaCl, 78 mM MgCl2, 50µg/ml propidium iodide | 2.4.2 |

**Table 2.6 – Summary of buffers and solutions**

# Chapter 3. Results: Nur1 dephosphorylation confers positive feedback to Cdc14 activation

As discussed in Chapter 1, a role for the Cdk-opposing phosphatase Cdc14 in controlling rDNA condensation and segregation is established, however which proteins Cdc14 is targeting to control this process remains unclear. Therefore, my initial approach was to try and identify Cdc14 targets that could be important for the accurate completion of rDNA condensation and/or segregation.

These targets will likely be dephosphorylated in anaphase, when these processes occur. With this in mind, I reviewed the results of a phosphoproteomics-based screen for novel Cdc14 targets in budding yeast mitotic exit (carried out by Thomas Kuilman) for targets which met our criteria (Kuilman et al., 2015). This screen exploited the fact that Cdc14 targets are dephosphorylated in an ordered manner, relative to the ratio of kinase/phosphatase activity in the cell. Briefly, cells were arrested in metaphase using an *S. cerevisiae* strain in which Cdc20, a subunit of the APC/C complex necessary for the progression from metaphase to anaphase, is not expressed. In these conditions Cdk activity is maintained at a constant level and endogenous Cdc14 is sequestered in the nucleolus. In this metaphase arrest Cdc14, itself under control of a GAL-inducible promoter, was expressed. This forced cells to undergo some aspects of mitosis, targets being dephosphorylated depending on the increasing activity of the phosphatase. For several examples, the order of dephosphorylation of Cdc14 targets mirrored that in a normal cell cycle. By using mass spectrometry to measure the disappearance of Cdk-phosphorylated phosphopeptides, this approach led to the identification of various novel Cdc14 targets (Kuilman et al., 2015).

As a candidate protein that could be involved in the regulation of rDNA, we selected the nuclear envelope protein Nur1 for further study. Along with its main binding partner Src1/Heh1, this protein has been designated a “Chromosome Linkage INM Protein”. It has been demonstrated to be important for the tethering of the rDNA and telomeres to the nuclear envelope, its absence leading to a decrease in rDNA repeat

stability and unequal rDNA segregation as well as loss of telomeric stability and telomere silencing (Mekhail, Seebacher, Gygi and Moazed, 2008; Chan et al., 2011; Poon and Mekhail, 2011). Further, this protein was previously identified as a Cdk target in a proteome wide study (Ubersax et al., 2003). Nur1 is predicted to have nine Cdk sites (two full and seven minimal sites), four of which have also been identified in mass spectrometry studies (Holt et al., 2009; Kuilman et al., 2015). It has two predicted helical trans-membrane domains, as well as a putative C-terminal nuclear localisation signal (Fig. 1A).

## 3.1 Nur1 is a Cdc14 target in anaphase

Of the four Nur1 Cdk sites confirmed by mass spectrometry, T. Kuilman's phosphoproteome analysis covered three phosphorylation sites on two phosphopeptides. These disappeared with early to intermediate timing, relative to the phosphopeptides of all detected proteins, during Cdc14 induced mitotic exit (Fig. 3.1B). This is compatible with the protein being dephosphorylated in anaphase.

In order to confirm that the protein was indeed dephosphorylated at this cell cycle stage *in vivo*, in a Cdc14-dependent manner, I carried out cell cycle analysis of the phosphorylation of the protein by looking for mobility shifts on a Phos-tag acrylamide gel. Cells were arrested in G1 by pheromone α-factor treatment, released at 35.5°C to progress through the cell cycle, and either rearrested in the following G1 in a wild type situation, or arrested at mitotic exit in a *cdc14-1* strain lacking Cdc14 activity at the restrictive temperature. Progression through the cell cycle was monitored by FACS analysis of DNA content.

In the wild type, Nur1 became phosphorylated by S phase, as evidenced by the appearance of slower migrating isoforms of the protein (Fig. 3.1C), Faster migrating isoforms of the protein reappeared 60-75 minutes after release from G1, consistent with the protein being dephosphorylated during anaphase, before cells completed cytokinesis at around 90 minutes (Fig. 3.1C).

In the *cdc14-1* background, after Cdc14 inactivation, the faster migrating forms of Nur1 never reappeared, indicating that Nur1 is not dephosphorylated in this case (Fig. 3.1C). To confirm that Cdc14 is directly responsible for dephosphorylating Nur1, and that the mobility shift observed on the gel is indeed due to phosphorylation, Nur1 was immunoprecipitated from mitotic cells arrested by treatment with nocodazole. The immunoprecipitated protein was then incubated either in a control buffer, or in the presence of commercially available λ phosphatase, or purified recombinant Cdc14 (a gift from C. Bouchoux)(Bouchoux and Uhlmann, 2011). Incubation with either phosphatase, but not the control buffer, led to the conversion of Nur1 from the slower migrating to the faster migrating species, confirming that the slower migrating forms are the consequence of phosphorylation (Fig. 3.1D).

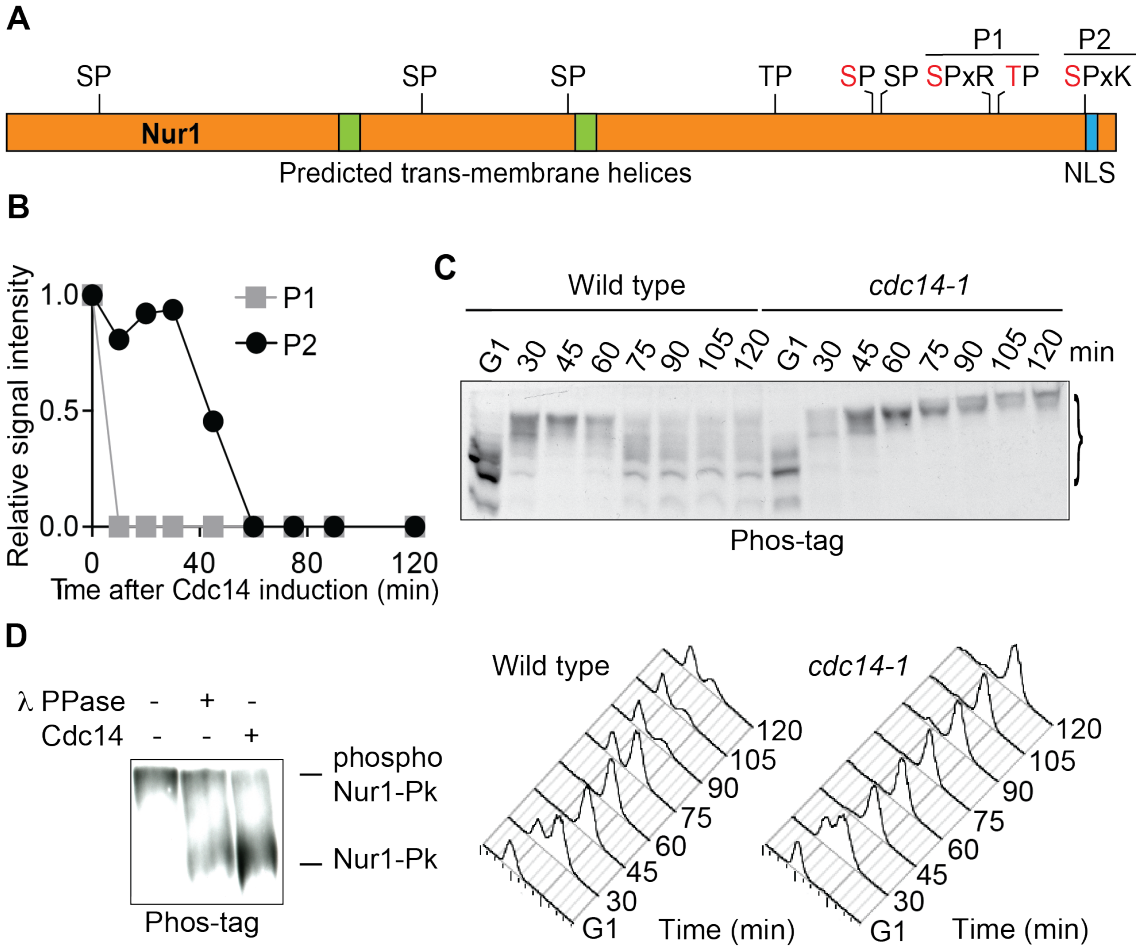



**Figure 3.1 – Nur1 dephosphorylation by Cdc14 in anaphase.**
**A**. Schematic representation of Nur1. Putative (black) and confirmed (red) Cdk phosphorylation sites, as well as other predicted landmarks, are indicated. P1, SLpSPLRKpTPLSAR; P2, NDINSILRpSPK. **B**. Nur1 phosphopeptide abundance over the course of Cdc14-induced mitotic exit. **C.** Nur1 phosphorylation-dependent mobility shifts during synchronous cell cycle progression at 35.5°C, in wild-type and *cdc14-1* cells, were analyzed using Phos-tag gels and Western blotting. Cell cycle progression was monitored by FACS analysis of DNA content. **D.** *In vitro* dephosphorylation of Nur1 by Cdc14. Immunopurified Nur1 was subjected to the indicated phosphatase or control treatments and analyzed by Phos-tag gel electrophoresis.

## 3.2 Nur1 dephosphorylation by Cdc14 is required for cell survival at high temperatures

In order to examine the importance of Nur1 dephosphorylation, we created a strain in which Nur1 is fused to Clb2 in an inducible fashion (technique adapted from (Lyons and Morgan, 2011)). This strategy relies on the integration of Clb2 after the gene of interest, between which a marker is situated. The marker is flanked by LoxP sites and can be removed through the action of CRE-ER, itself activated upon the addition of β-estradiol, leading to the transcription of the fusion protein (Fig. 3.2A). Due to the presence of Clb2, Cdc28 will be permanently recruited to Nur1 and lead to hyperphosphorylation of the protein. As a control, a version of Clb2 harbouring three point mutations that prevent it from binding to and recruiting Cdc28 was also fused to Nur1 (Nur1-Clb2ΔCdk fusion). The replacement of the endogenous protein with the larger fusion protein is an efficient process and complete within 4 hours of induction. The Nur1-Clb2 fusion protein has a smeary aspect on a western, indicative of the presence of slower migrating forms characteristic of protein phosphorylation. This smear or haze was not present in the Nur1-Clb2ΔCdk fusion (Fig. 3.2C).

It was next examined what effect the fusions would have on cell survival. Strikingly, the Nur1-Clb2 fusion was viable at 25°C but unable to grow at the higher temperature of 36°C, whereas the Nur1-Clb2ΔCdk fusion was viable at both temperatures. This approach, however, does not take into account the possibility that the phenotype could be caused by Cdc28 phosphorylating other proteins in the vicinity of Nur1, having been attracted to the nucleolus in higher than usual levels, or phosphorylating non-consensus sites within the protein. In order to control for these scenarios, we created a version of Nur1 in which the 9 putative Cdk sites were replaced by alanines (Nur1(9A)). The process of generating Nur1(9A)-Clb2 and Nur1(9A)-Clb2ΔCdk fusions was then repeated.

This mutation of the 9 Cdk sites completely rescued the lethality of the Nur1-Clb2 fusion (Fig. 3.2B). It also prevented the mobility shift following Nur1-Clb2 fusion (Fig. 3.2D). This suggests that indeed persistent Nur1 phosphorylation on its Cdk phosphorylation sites is the cause for a temperature sensitive growth defect.

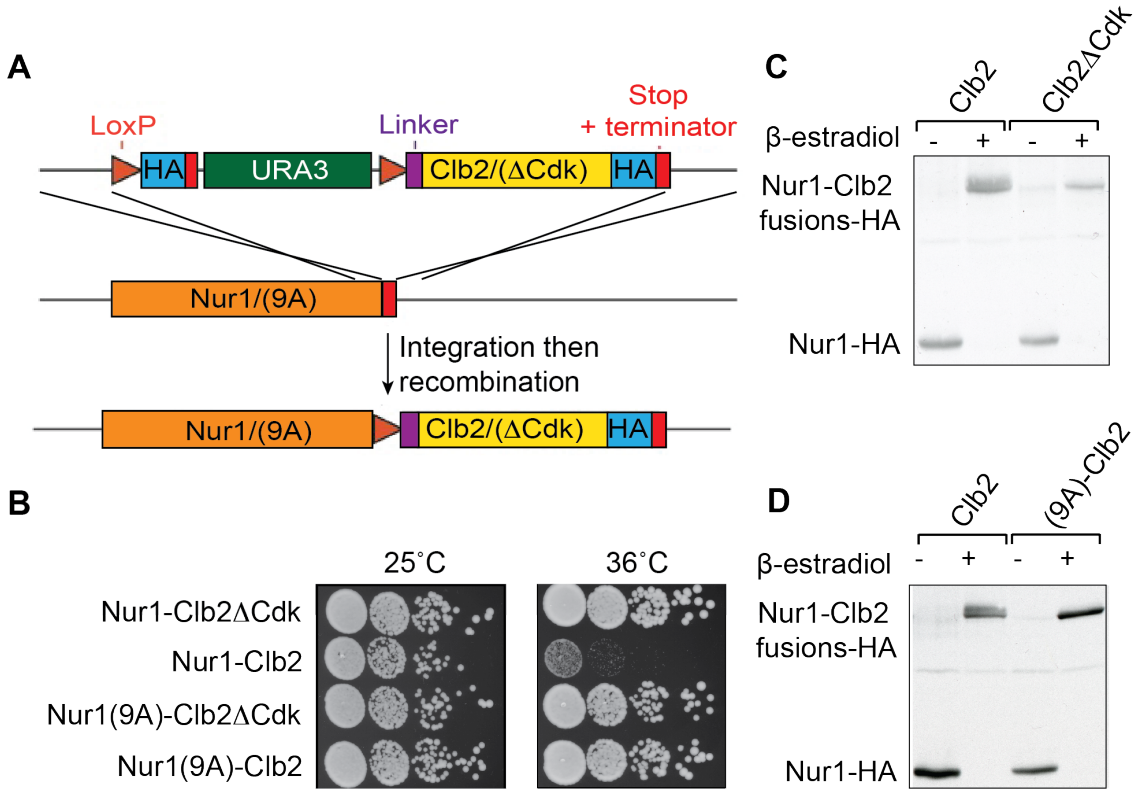



**Figure 3.2 – Creation of an inducible Nur1-Clb2 fusion causes cell death at high temperatures**

**A.** Design scheme of the Nur1-Clb2 fusion strains. **B.** Western blot demonstrating replacement of endogenous Nur1 with Nur1-Clb2, or Nur1-Clb2ΔCdk, 4 hours after β-estradiol addition. **C.** Serial dilution assay to compare survival of Nur1-Clb2, Nur1-Clb2ΔCdk, Nur1(9A)-Clb2 and Nur1(9A)-Clb2ΔCdk-expressing cells, at 25°C and 36°C. **D.** Western blot demonstrating replacement of Nur1 or Nur1(9A) with Nur1-Clb2 and Nur1(9A)-Clb2, respectively, 4 hours after β-estradiol addition.

## 3.3 Nur1 dephosphorylation promotes timely rDNA segregation

### 3.3.1 Phosphorylated Nur1 delays rDNA segregation

To study the effect of permanent phosphorylation of Nur1 on rDNA segregation, Net1, an abundant rDNA binding protein, was visualised by tagging with YFP and performing indirect immunofluorescence, in order to monitor the behaviour of the rDNA. Cells were synchronised in G1 by pheromone α-factor treatment, released to go through the cell cycle at 36°C, and the timing of rDNA segregation was monitored as the cells progressed through mitosis. The segregation of the rDNA – whether or not the rDNA had separated into two halves at opposite poles of the cell – was quantified relative to the length of the anaphase spindle, as an internal control for progression through anaphase.

Both in a wild type strain and in the Nur1-Clb2ΔCdk control strain, complete rDNA segregation was initially recorded at a spindle length of 5-6 μm and was observed in 100% of cells by the time spindles reached 8-9 μm. Conversely, in the Nur1-Clb2 strains, complete rDNA segregation was only observed from spindle lengths of 7-8 μm, and even when the spindle was fully elongated, at a length of 9-10 μm or more, rDNA segregation was still not complete in all cells at 36°C (Fig. 3.3A). A defect in rDNA segregation was also observed, although less pronounced, at 25°C (Fig. 3.4). Observations of the condensation state of the rDNA by immunofluorescence revealed that the wild-type or Nur1-Clb2ΔCdk control showed the appropriate highly condensed form for this locus in anaphase, neatly segregated to two the poles of the dividing cell. In contrast, in the Nur1-Clb2 strain the rDNA appeared more uncondensed, and often stretched out between the two cells across the bud neck (Fig. 3.3B). From these observations it can be concluded that constitutive phosphorylation of Nur1 leads to defective rDNA segregation.

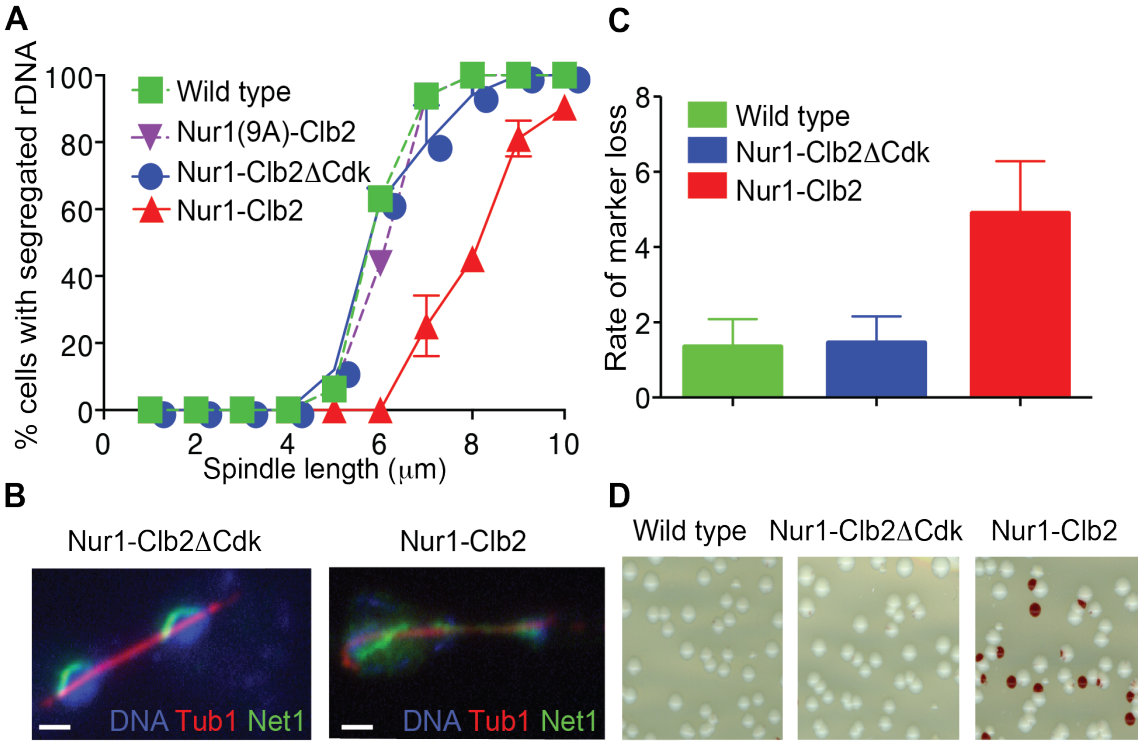



**Figure 3.3 – Nur1-Clb2 delays rDNA segregation**

**A.** rDNA segregation as a function of spindle length, compared between Nur1-Clb2 and Nur1-Clb2ΔCdk-expressing strains. At least 15, but typically more, cells were scored for each spindle length category. The mean and standard deviation from three independent experiments is shown. Wild type and Nur1(9A)-Cdk strains were also included in one repeat of the experiment. **B.** Representative images of rDNA segregation in a Nur1-Clb2ΔCdk cell, and of an anaphase bridge formed of rDNA in a Nur1-Clb2 cell. The rDNA was visualized by the rDNA binding protein Net1, fused to YFP, the spindle was detected with an antibody against α-tubulin, DNA was stained with 4',6-diamidino-2-phenylindole (DAPI). **C.** ADE2 marker loss from the rDNA repeats is shown as a percentage of half red-sectored colonies after plating the indicated strains, with **D.** A representative field of colonies shown for each genotype. Around 500 colonies were scored for each strain. The mean and standard deviation from four independent experiments is shown.

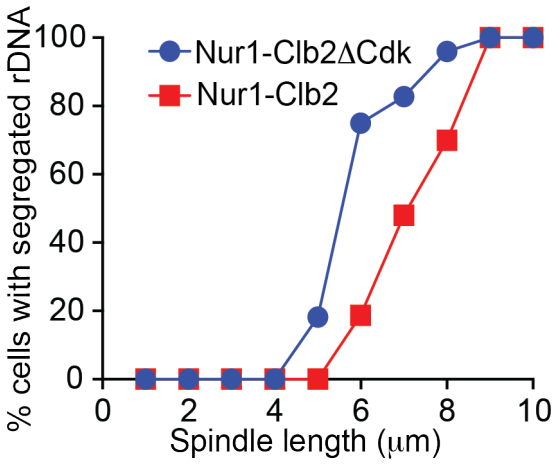



**Figure 3.4 – rDNA segregation is delayed in Nur1-Clb2 at 25°C**
rDNA segregation as a function of spindle length, compared between Nur1-Clb2 and Nur1-Clb2ΔCdk-expressing strains. At least 15, but typically more, cells were scored for each spindle length category.

A possible consequence of defective rDNA segregation is unequal segregation of this locus between the mother and daughter cells. To measure rDNA stability, we quantified the rate of loss of an *ADE2* marker integrated within the rDNA repeats. Loss of *ADE2* causes accumulation of a red intermediate metabolite in the adenine synthesis pathway, leading to the growth of red colonies when cells are plated on low adenine media. *ADE2* loss during the first cell division after plating will generate half red-sectored colonies. The Nur1-Clb2 fusion strains have a significant increase in the fraction of half-sectored colonies, compared to the Nur1-Clb2ΔCdk or wild-type strains. This indicates that there is not only a delay in rDNA segregation, but also mis-segregation of the repeats in a certain fraction of the affected cells (Fig. 3.3C-D).

### 3.3.2 Cdc14 overexpression rescues the rDNA segregation delay

To confirm that it is indeed lack of dephosphorylation by Cdc14 that is responsible for the delay in rDNA segregation, an extra copy of Cdc14 was integrated in the Nur1-Clb2 strain and the Nur1-Clb2ΔCdk control strain, under the control of a galactose-inducible promoter (GAL-CDC14). In an experiment in which cells were arrested in G1 by α-factor pheromone treatment and released to progress through the cell cycle, overexpression of Cdc14 from metaphase onwards led to disappearance of the slower migrating forms of Nur1-Clb2, indicating that the protein may be dephosphorylated (Fig. 3.5A). Monitoring the segregation of the rDNA relative to the length of the anaphase spindle, as previously, it was found that Cdc14 overexpression rescued the rDNA segregation delay observed in the Nur1-Clb2 fusion.

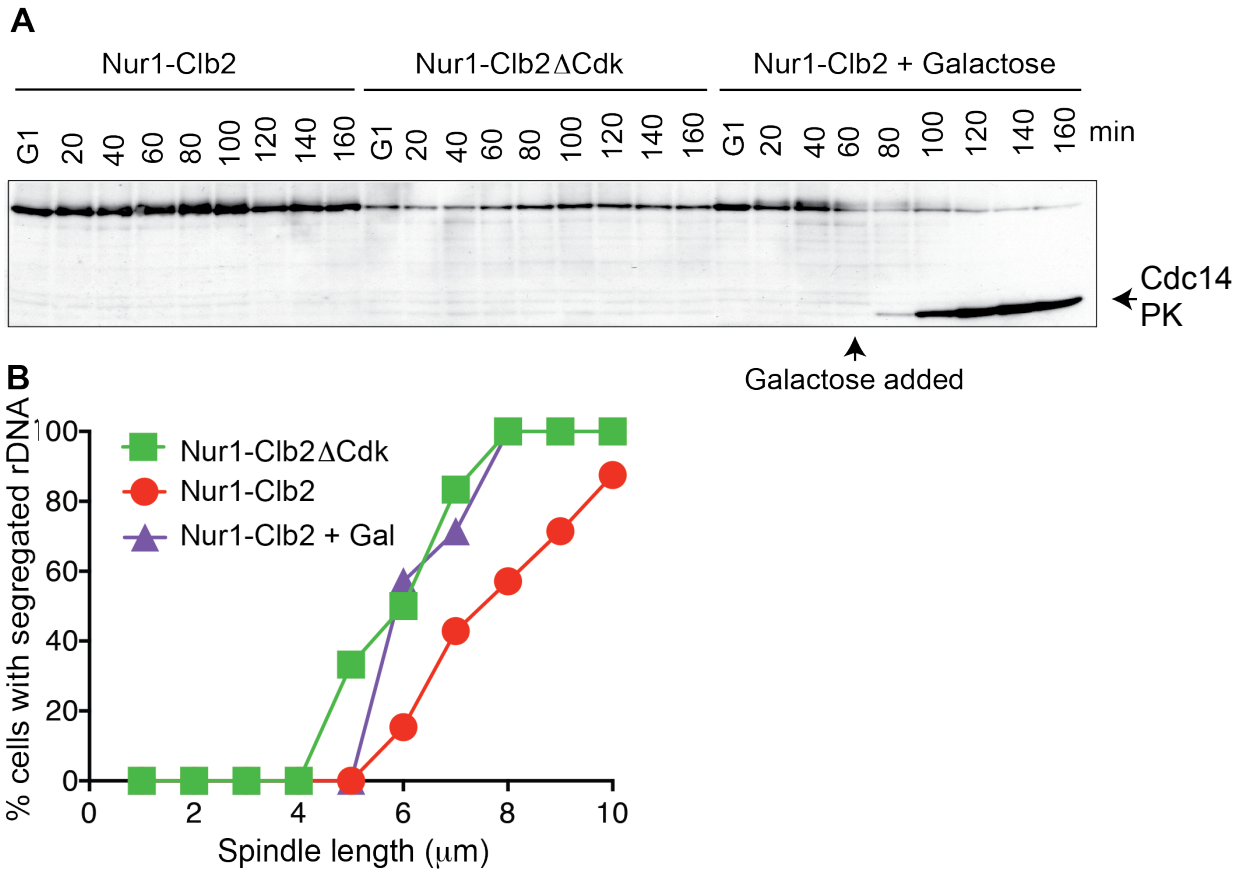



**Figure 3.5 – Cdc14 overexpression rescues rDNA segregation defect in the Nur1-Clb2 fusion strain.**

**A.** Overexpression of Cdc14 from a Galactose inducible promoter from metaphase onwards leads to dephosphorylation of Nur1-Clb2. Cells were grown in YP+raffinose and galactose was added 60 minutes after release from G1. The arrow in the right indicates Cdc14-PK expression. **B.** rDNA segregation in the Nur1-Clb2 strain was rescued by overexpression of Cdc14, in the same experiment.

## 3.4 Persistent Nur1 phosphorylation delays mitotic progression

Given Nur1's role as a nuclear rim protein and its direct contacts with rDNA-binding proteins, one possibility is that Nur1 plays a direct role in regulating the condensation status of the rDNA, influencing its ability to segregate correctly. On the other hand, delayed rDNA segregation is a hallmark of defects in mitotic exit, for instance being a characteristic phenotype of FEAR pathway mutants, so a second possibility is that the phenotype observed is a consequence of a higher level defect in the process of mitotic exit itself.

In order to test this hypothesis, cells were arrested in G1 by α-factor treatment, released to progress through the cell cycle in a synchronous manner at 36°C, and rearrested in the following G1, during which experiment the timing of progression through mitosis was monitored. FACS analysis showed that Nur1-Clb2 cells spent at least 20 minutes longer with a 2C DNA content than a Nur1-Clb2ΔCdk control, indicative of a delay somewhere in mitosis (Fig. 3.6A). To investigate in more detail at which stage the delay was occurring, several cell cycle markers were examined by Western blot at frequent intervals throughout the cell cycle. The timing of appearance of Clb2 and Securin (S phase) was identical between Nur1-Clb2 and the Nur1-Clb2ΔCdk control, as was the similarly timed phosphorylation of Orc6. However, the disappearance of both Clb2 and Securin were markedly delayed, as were the dephosphorylation of Orc6 during mitotic exit and re-accumulation of the Cdk inhibitor and G1 marker, Sic1 (Fig. 3.6B). To distinguish whether the delay took place before or after the metaphase-anaphase transition, the proportion of metaphase spindles (1-3 μm) versus anaphase spindles (>3 μm) was quantified at 10-minute intervals as cells progressed synchronously through mitosis. There was a slight delay in progression through metaphase, but more strikingly a long prolongation of the time that Nur1-Clb2 cells spent with anaphase spindles (Fig. 3.6C). These observations are consistent with a delay of Nur1-Clb2 cells both at the metaphase to anaphase transition as well as during mitotic exit. A similar delay in mitotic progression, although less pronounced, was observed in the same experiment done at 25°C (Fig. 3.7).

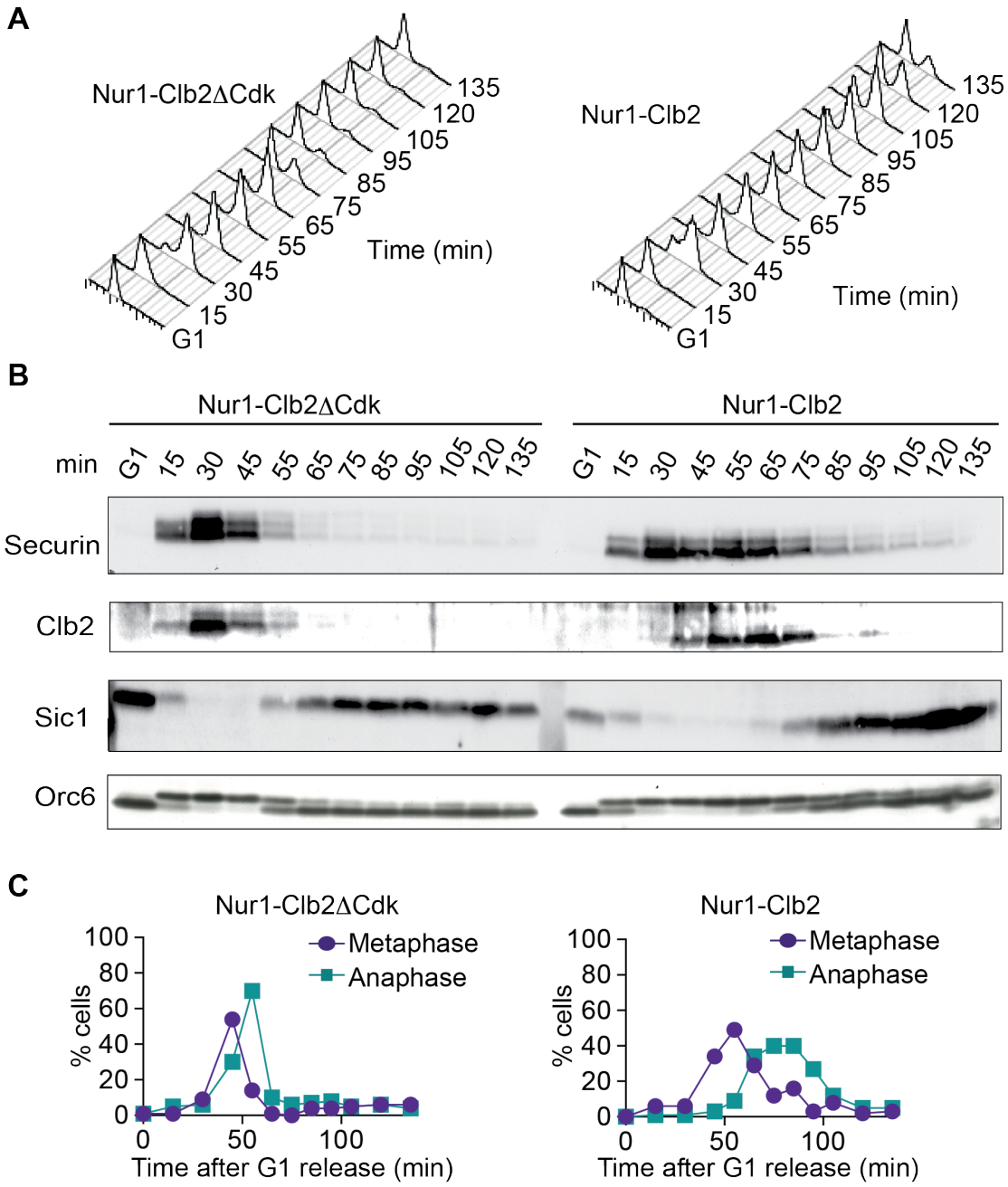



**Figure 3.6 – Nur1-Clb2 causes a mitotic exit defect at 36°C**
Nur1-Clb2 and Nur1-Clb2ΔCdk cells were synchronized in G1 by a-factor treatment and released to progress through the cell cycle at 36°C, before being rearrested in the following G1. At time points throughout the cell cycle we monitored, **A.** Cell cycle progression by FACS analysis of DNA content. **B.** Western blot analysis of the cell cycle markers securin, Clb2, Sic1 and Orc6, and **C.** The percentages of cells displaying metaphase (1–3 mm) or anaphase (≧3 mm) spindles were scored. 100 cells were counted at each time point.

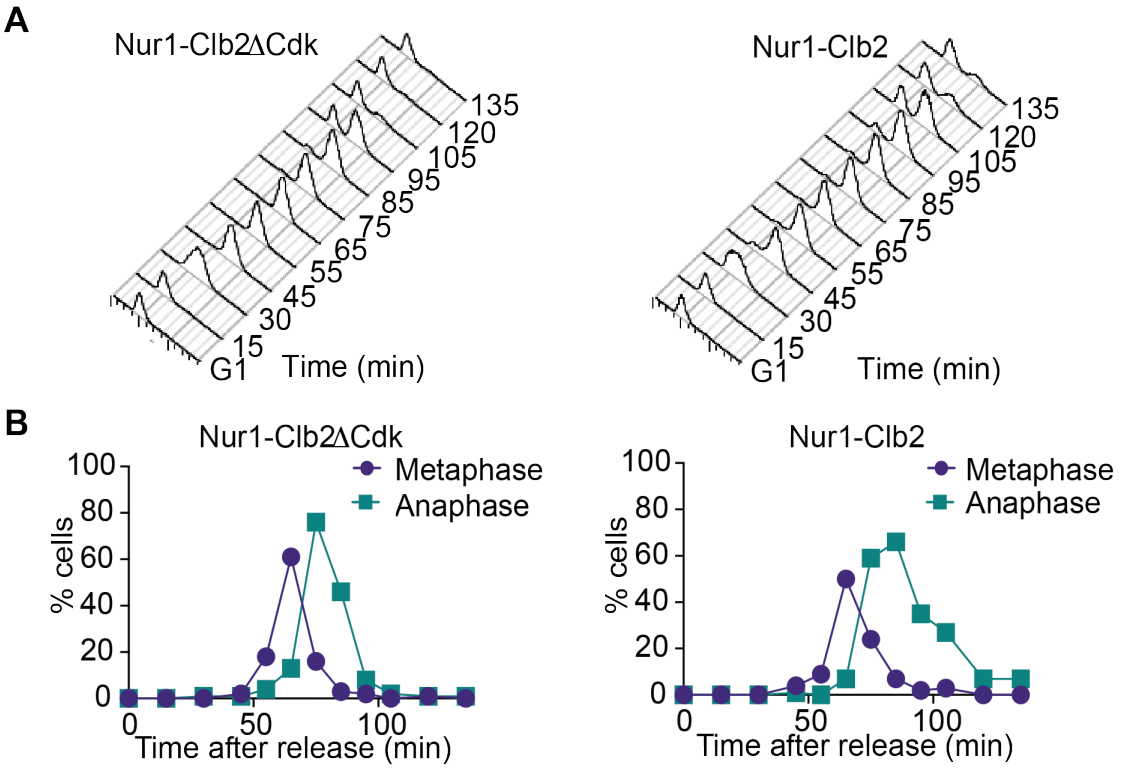



**Figure 3.7 – Nur1-Clb2 causes a mitotic exit defect at 25°C**
Nur1-Clb2 and Nur1-Clb2ΔCdk cells were synchronized in G1 by a-factor treatment and released to progress through the cell cycle at 25°C, before being rearrested in the following G1. At time points throughout the cell cycle we monitored, **A.** Cell cycle progression by FACS analysis of DNA content and **B.** The percentages of cells displaying metaphase (1–3 mm) or anaphase (≧3 mm) spindles were scored. 100 cells were counted at each time point.

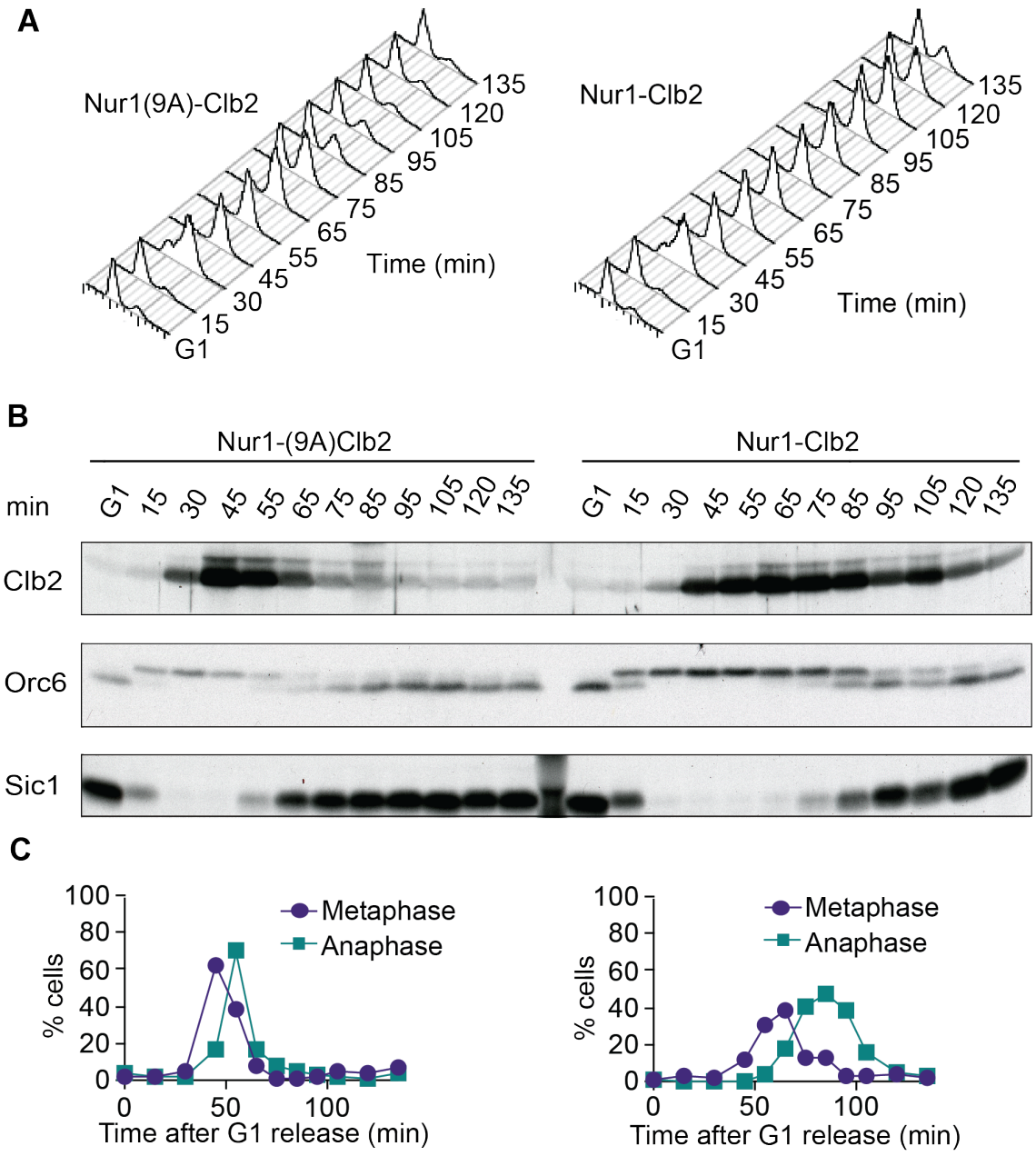



**Figure 3.8 – Nur1 phosphorylation is responsible for the mitotic exit delay seen in Nur1-Clb2 cells**

Nur1-Clb2 and Nur1-(9A)Clb2 cells were synchronized in G1 by a-factor treatment and released to progress through the cell cycle at 36°C, before being rearrested in the following G1. At time points throughout the cell cycle we monitored, **A.** Cell cycle progression by FACS analysis of DNA content. **B.** Western blot analysis of the cell cycle markers Clb2, Sic1 and Orc6, and **C.** The percentages of cells displaying metaphase (1–3 mm) or anaphase (≧3 mm) spindles were scored. 100 cells were counted at each time point.

To confirm that the delay was due to constitutive phosphorylation of Nur1, we repeated the same experiment using the Nur1(9A)-Clb2 fusion as a control instead of Nur1-Clb2ΔCdk. We found that the Nur1(9A)-Clb2 completely rescued all aspects of mitotic progression – according to FACS analysis, western blot of cell cycle markers, and monitoring of spindle morphology (Fig. 3.8).

This leads us to conclude that the delay in rDNA segregation is likely due to a higher-level delay in mitotic exit itself, for which constitutive Nur1 phosphorylation is responsible.

## 3.5 Phospho-Nur1 plays a role in sequestering Cdc14

### 3.5.1 Nur1-Clb2 delays Cdc14 release

The mitotic exit delay in Nur1-Clb2 fusion cells is reminiscent of that seen in FEAR pathway mutants, in which a delay in activation of the Cdc14 phosphatase leads to delayed mitotic exit and rDNA segregation. Given the similarities in phenotype between mutants with delayed Cdc14 release and our Nur1-Clb2 fusion mutant strain, I decided to investigate the timing of Cdc14 release in the latter. Again, cells were synchronized in G1 and the timing of Cdc14 release was measured as cells passed through mitosis. As with the investigation of rDNA segregation, release of Cdc14 was quantified relative to the length of the mitotic spindle, as an internal control for the timing of progression through mitosis.

I found over several experimental repeats that there was a small but statistically significant delay ($p<0.005$) in the timing of Cdc14 release from the nucleolus in the Nur1-Clb2 fusions as compared to the Nur1-Clb2ΔCdk control (Fig. 3.9). This indicates that phosphorylated Nur1 is impeding Cdc14 release, which in turn would be responsible for the delay in mitotic exit and rDNA segregation.

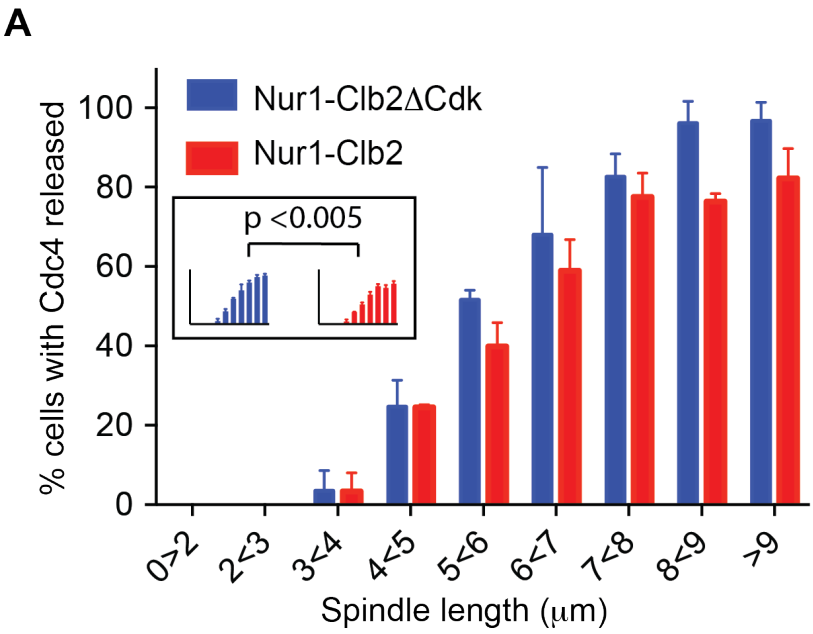



**Figure 3.9 – Nur1-Clb2 delays Cdc14 release**
Quantification of Cdc14 release, in experiments performed as in Figure 3.6, relative to spindle length. At least 15, but typically more, cells were counted for each spindle length category. The mean and standard deviation from 3 independent experiments is shown. A logistic regression analysis showed that Cdc14 release timing in the Nur1-Clb2 strain was significantly delayed.

### 3.5.2 *CDC14$^{TAB6-1}$* rescues survival and mitotic progression of Nur1-Clb2

If phosphorylated Nur1 is preventing Cdc14 from being released, then inserting the dominant active *CDC14$^{TAB6-1}$* allele, which leads to Cdc14 being less tightly bound by Net1, should restore cell survival at high temperature. This allele was able to completely rescue growth of Nur1-Clb2 at 36°C (Fig. 3.10A). As a control, it was confirmed that *CDC14$^{TAB6-1}$* did not cause dephosphorylation of Nur1-Clb2, due to the presence of higher than normal levels of Cdc14 activity. Western blotting revealed that Nur1-Clb2 mobility, in particular its slower migrating forms, is unaffected by the presence of *CDC14$^{TAB6-1}$* (Fig. 3.10B). Therefore the temperature sensitive growth due to persistent Cdk phosphorylation of Nur1 is caused by defective Cdc14 activation.

Progression through the cell cycle was monitored in Nur1-Clb2 with and without *CDC14$^{TAB6-1}$*. FACS analysis and Western blotting of the cell cycle markers Clb2 and Sic1 revealed that normal cell cycle progression was largely restored, with the delay at the metaphase to anaphase transition and during mitotic exit being rescued (Fig. 310C-D). This was further confirmed by monitoring of spindle morphology, where it was noted that normal progression through mitosis was restored (Fig. 3.10E).

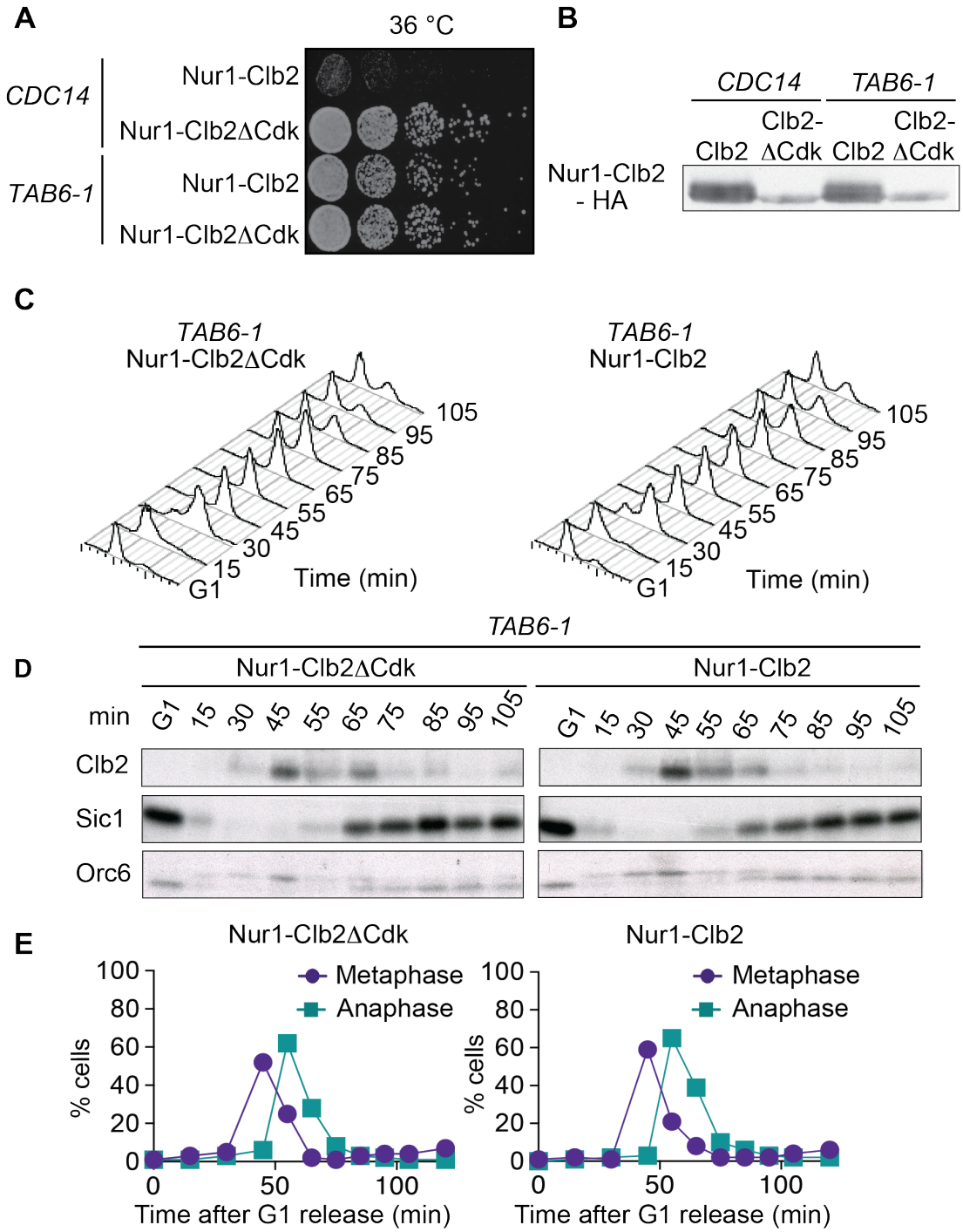



**Figure 3.10 – Cdc14$^{TAB6-1}$ rescues Nur1-Clb2**
**A.** Serial dilution assay demonstrating rescue of cell survival of Nur1-Clb2 cells by dominant active Cdc14$^{TAB6-1}$. **B.** Western blot samples taken of the cells in B, before they were plated, shows that Cdc14$^{TAB6-1}$ did not alter the Nur1-Clb2 phosphorylation status. **C.-E.** Active Cdc14 rescues mitotic progression of Nur1-Clb2 cells. As in Figure 3.6, but strains carried the CDC14$^{TAB6-1}$ allele.

We can conclude from these experiments that Nur1, and more specifically phosphorylated Nur1, is preventing Cdc14 from being properly released in early anaphase, thus causing defective mitotic exit. If this is the case, it can be postulated that either eliminating Nur1 or removing its Cdk phosphorylation sites should therefore facilitate Cdc14 release.

## 3.6 Phospho-Nur1 counteracts Cdc14 release in early anaphase

### 3.6.1 *nur1Δ* or *nur1(9A)* cause premature Cdc14 release

To test this hypothesis, a detailed study into the timing of Cdc14 release, looking both at partial (with Cdc14 still enriched in the nucleolus but also present in the nucleus and/or cytoplasm) and full release, was carried out. Again, G1-G1 experiments were performed in which wild-type, *nur1Δ,* and *nur1(9A)* strains were compared, with Cdc14 release state related to spindle length.

Three independent experiments corroborated the fact that Cdc14 was indeed released both earlier and more abruptly in cells lacking Nur1. Strikingly, Cdc14 was detected, either partially or fully released, in more than half of *Δnur1* or *nur1(9A)* cells at spindle lengths below 3 μm, when Cdc14 release is almost never seen in wild type cells. Even in metaphase cells with short (1-2 μm) spindles, when Cdc14 is usually kept tightly sequestered in the nucleolus, Cdc14 release was observed in a small proportion of *Δnur1* or *nur1(9A)* cells (Fig. 3.11A). Representative images are shown of metaphase cells, in wild-type strains with Cdc14 sequestered in the nucleolus, and in *nur1Δ* with Cdc14 fully released (Fig. 3.11B).

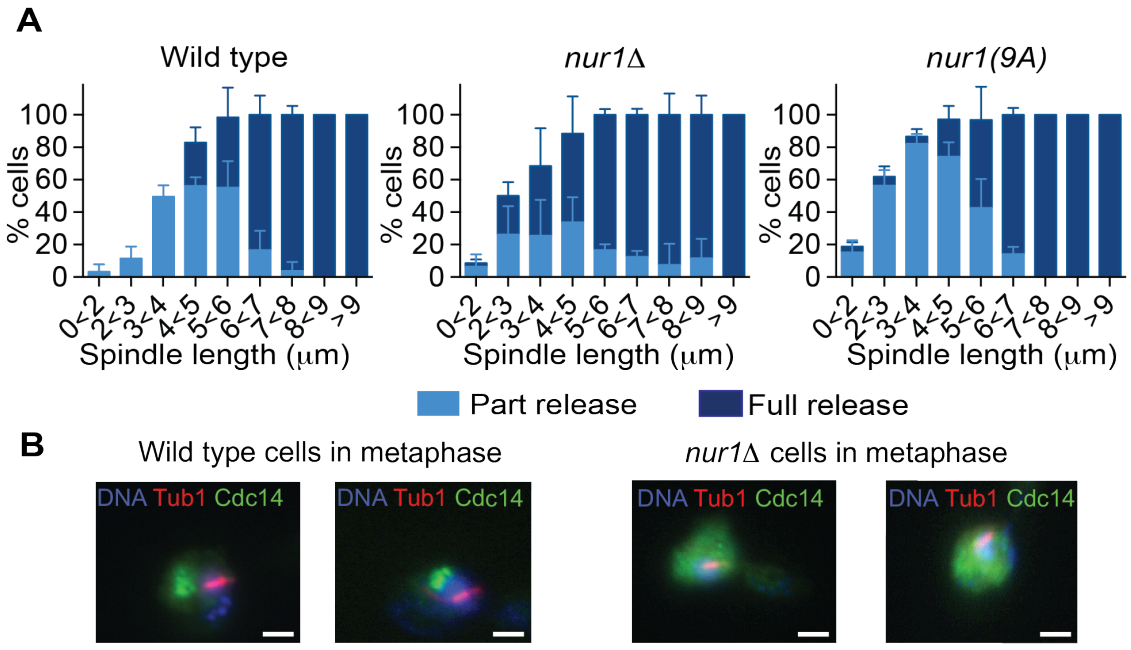



**Figure 3.11 – Absence of Nur1 or of its Cdk phospho-sites leads to early Cdc14 release**
**A.** Quantification of Cdc14 release versus spindle length, as in Fig. 3.9, in wild type, *nur1Δ* and *nur1(9A)* cells. Cdc14 release was subdivided into 'Partial release', when Cdc14 was detectably released into the nucleus but some nucleolar enrichment persisted and 'Full release', when no nucleolar Cdc14 enrichment remained detectable. The mean and standard deviation from three independent experiments is shown. **B.** Images of Cdc14 in wild type and *nur1Δ* cells. The metaphase state is confirmed by the presence of a short spindle, stained with an a-tubulin antibody. Cdc14-GFP was detected with an a-GFP antibody, DNA was counterstained with DAPI.

### 3.6.2 Premature Cdc14 release does nor affect the Spindle Assembly Checkpoint

Early Cdc14 release could in theory lead to defective activation of the Spindle Assembly Checkpoint (SAC). Indeed, one of the early anaphase roles of Cdc14 is to inactivate the checkpoint by dephosphorylating Sli15 (forming part of the chromosome tension sensor) (Mirchenko and Uhlmann, 2010). If Cdc14 were released too early, Sli15 could already be dephosphorylated, leading to an inability of cells to respond to checkpoint defects. To test whether this was the case, we examined *nur1Δ* cells' sensitivity to the microtubule depolymerising drug benomyl – if cells are unable to activate the SAC, they should die in the presence of this drug, as is the case in a *mad2Δ* background.

However, we found that *nur1Δ* cells behave as wild type when grown in the presence of benomyl (Fig. 3.12). We hypothesise that while Cdc14 is released early in *nur1Δ* and *nur1(9A)* cells, at times before anaphase onset this amounts to a small increase in Cdc14 concentration that is probably not enough to shift the kinase to phosphatase ratio far enough to have any significant effect on the phosphorylation state of mitotic exit proteins.

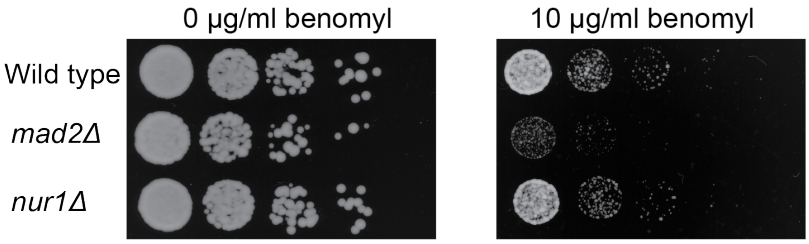



**Figure 3.12 – Early Cdc14 release does not cause SAC activation**
Serial dilution assay to compare survival of wild type, *mad2Δ* and *nur1Δ* cells in the presence of 10 µg/ml benomyl.

### 3.6.3 *nur1Δ* or *nur1(9A)* can rescue FEAR pathway mutants

In wild type, Cdc14 activation is promoted by the FEAR network in early anaphase. To further understand the role of Nur1 in Cdc14 release at this time, we introduced *nur1Δ* and *nur1(9A)* alleles into a *spo12Δ* strain, lacking a key component of the FEAR network (see Section 1.4). As previously, Cdc14 release was related to spindle length in synchronous cell cycle experiments, and it was quantified whether Cdc14 was partially or fully released.

In *spo12Δ* alone, Cdc14 release is severely delayed, with cells only showing released Cdc14 when spindles reached 6 µm. By introducing *nur1Δ* and *nur1(9A)* into this background, Cdc14 release was restored in early anaphase. The Cdc14 release profile in the *nur1Δ spo12Δ* and *nur1(9A) spo12Δ* double mutant strains was similar to that of wild type cells. However, at short spindle lengths, *nur1Δ spo12Δ* and *nur1(9A) spo12Δ* cells still displayed premature and more extensive Cdc14 release, as compared to wild type, while at longer spindle lengths the rescue did not fully reach wild type release levels (Fig. 3.13). These findings confirm that phosphorylated Nur1 is a strong inhibitor

of Cdc14 release in early anaphase and that the FEAR network acts to overcome Cdc14 inhibition by Nur1. However, the incomplete rescue of Cdc14 release in *spo12Δ* cells by Nur1 deletion also suggests that the FEAR network acts at least in part by a mechanism other than the inactivation of Nur1.

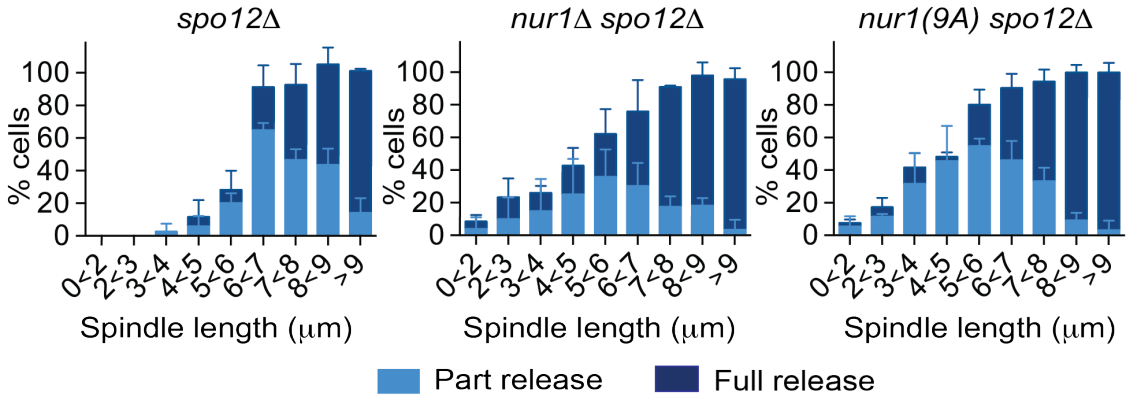



**Figure 3.13 – Preventing Nur1 phosphorylation restores Cdc14 release in a FEAR pathway mutant.**
Quantification of Cdc14 release versus spindle length, in *spo12Δ*, *nur1Δ spo12Δ* and *nur1(9A) spo12Δ* cells. As previously, Cdc14 release was subdivided into 'Partial release' and 'Full release'. The mean and standard deviation from three independent experiments is shown.

Next, the timing of cell cycle progression was compared between wild type, *nur1Δ* and *nur1(9A)* strains. Despite the early Cdc14 release in the latter strains, the timing of progression through mitosis, as measured by the fractions of cells displaying metaphase and anaphase spindles at each time point, was identical to wild type (Fig. 3.14). As previously reported, in a *spo12Δ* background, anaphase was extended by around 20 minutes. In this case, *nur1Δ* or introduction of *nur1(9A)* led to a near complete rescue of the kinetics of cell cycle progression (Fig. 3.14).

I subsequently investigated whether Nur1 plays a more global role in Cdc14 release or else specifically genetically interacts with Spo12. Both the experiments regarding Cdc14 release and mitotic progression were repeated in a *slk19Δ* background, defective for another protein involved in the FEAR pathway. As anticipated, Cdc14 release was delayed (although to a lesser extent than in the *spo12Δ* background) until later in

anaphase, when spindles had reached 5-6 µm, in the *slk19Δ* deletion alone. Further, a partial restoration of early Cdc14 release was observed in *nur1Δ slk19Δ*, similarly to what was observed in *spo12Δ* cells (Fig. 3.15A). Introduction of *nur1Δ* into *slk19Δ* background also led to a rescue of the delay in anaphase seen in the latter (Fig. 3.15B).

These results further confirm that Nur1, specifically in its phosphorylated form, can delay mitotic progression.

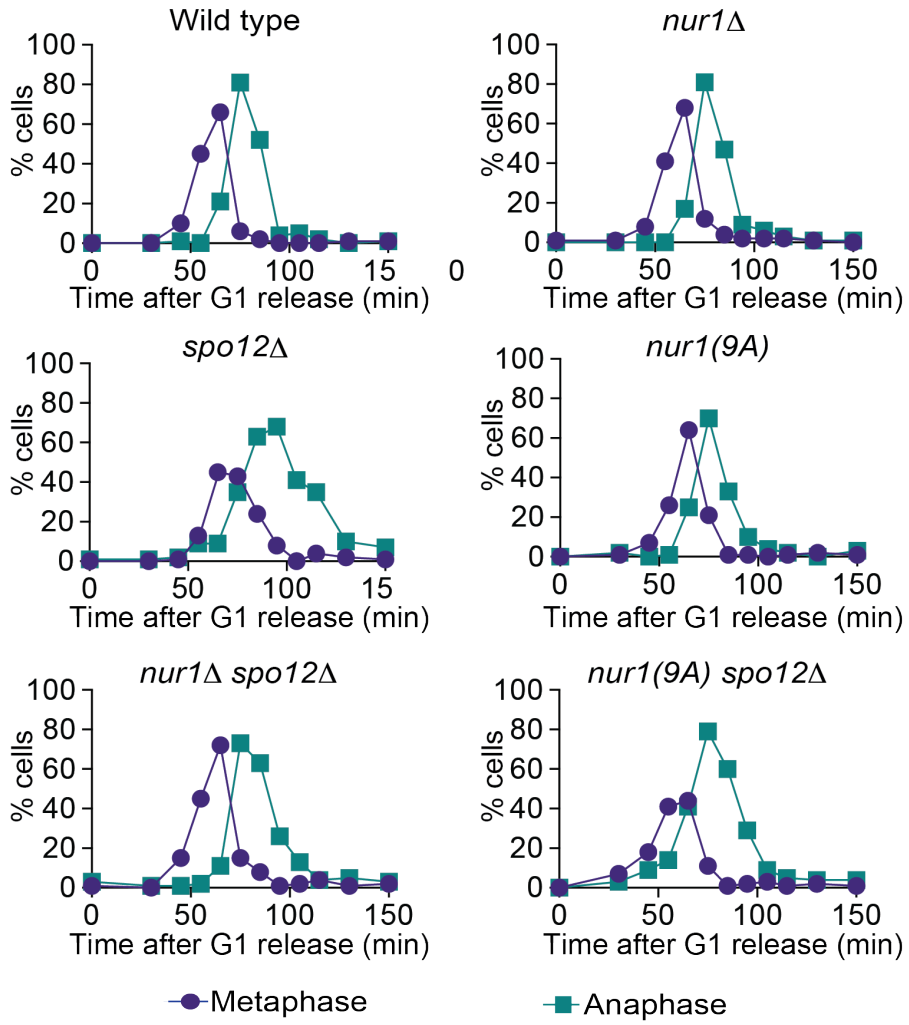



**Figure 3.14 – Preventing Nur1 phosphorylation restores timely progression through mitotic exit in a FEAR pathway mutant**
Progression through mitosis of in wild type, *nur1Δ*, *nur1(9A)*, *spo12Δ*, *nur1Δ spo12Δ* and *nur1(9A) spo12Δ* was measured by counting percentages of cells displaying metaphase (1–3 µm) or anaphase (>3 µm) spindles during synchronous cell cycle progression.

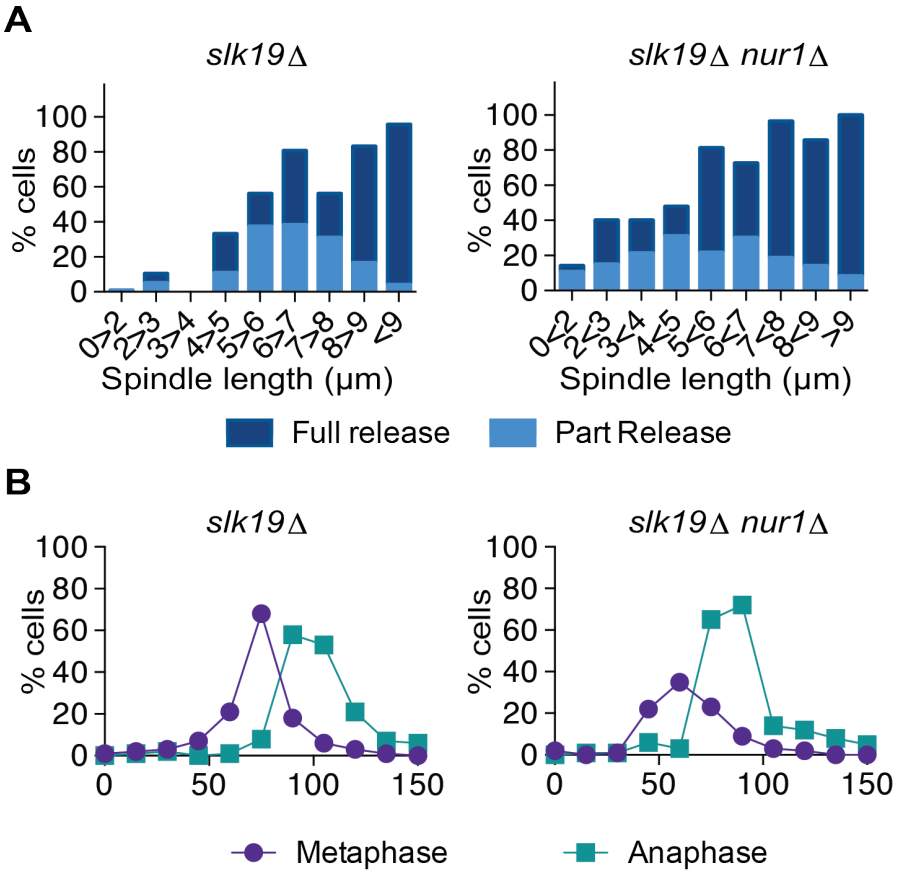



**Figure 3.15 – Nur1 deletion can rescue Cdc14 release and cell cycle progression in a second FEAR pathway mutant**

**A.** Quantification of Cdc14 release versus spindle length, as in Fig. 3.12, in *spo12Δ*, *nur1Δ slk19Δ* As previously, Cdc14 release was subdivided into 'Partial release' and 'Full release'. **B.** Progression through mitosis of the strains above was measured by counting percentages of cells displaying metaphase (1–3 μm) or anaphase (>3 μm) spindles during synchronous cell cycle progression.

## 3.7 Nur1 inactivation does not compensate for MEN defects

Inactivation of Nur1, by deletion or mutation of its Cdk phosphorylation sites, increases release of Cdc14 in early anaphase and almost completely rescues strains with defective FEAR pathway signalling. On the one hand, this could be because phospho-regulation of Nur1 has a role specific to early anaphase. On the other hand, Nur1 might be a general inhibitor of Cdc14 release, at all stages of mitotic exit.

To differentiate between these two possibilities, we tested whether Nur1 inactivation can also compensate for partial loss of MEN signalling. We used conditional thermosensitive alleles in two kinases active in the MEN pathway (see section 1.4), *cdc15-2,* and *dbf2-2,* and asked whether *nur1Δ* could rescue cell growth at a semi-permissive temperature with partially defective MEN signalling, as was observed for instance in *tof2Δ* mutants, where Cdc14 release is easier (Waples, Chahwan, Ciechonska and Lavoie, 2009). However, it was found that *cdc15-2 Δnur1* and *dbf2-2 nur1Δ* double mutants died to the same extent as the parental strains *cdc15-2,* and *dbf2-2,* as the temperature increased, whereas *Δnur1* strains alone were fully viable at all temperatures tested (Fig. 3.16).

Thus, Nur1 does not appear to act in late anaphase, when the MEN is controlling Cdc14 release. Instead, the function of Nur1 as a Cdc14 inhibitor seems to be restricted to early anaphase. This conclusion is consistent with the pattern of Nur1 phosphorylation during mitotic exit. Nur1 is phosphorylated by Cdk during early mitosis, when it counteracts Cdc14 release. In anaphase, Nur1 becomes dephosphorylated due to Cdc14 action, thus losing its inhibitory effect on Cdc14.

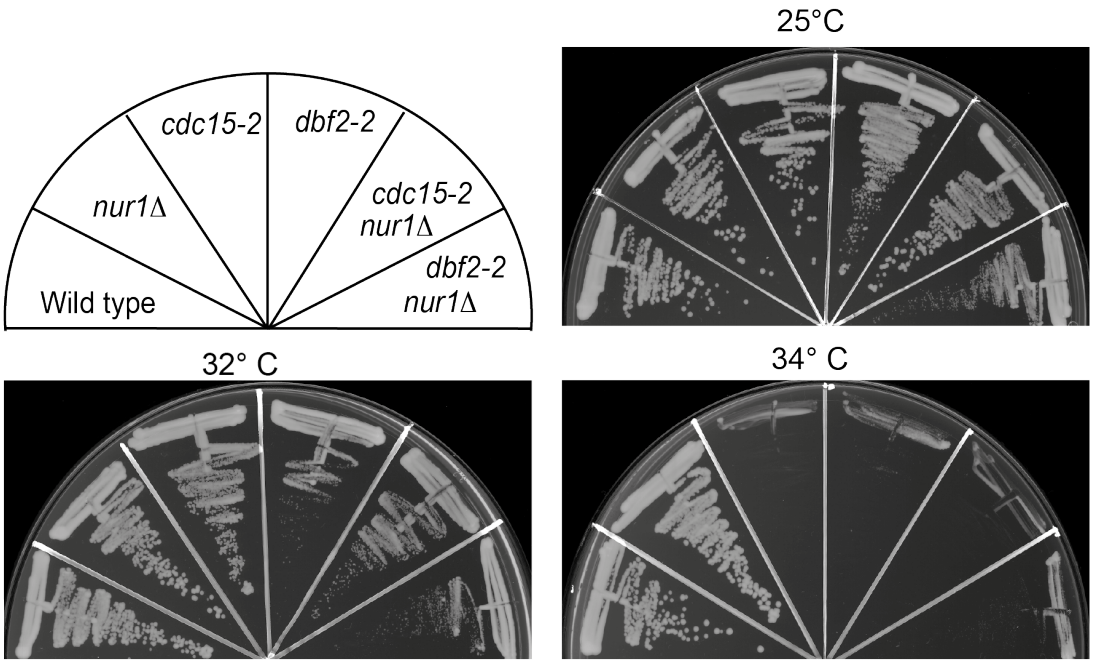



**Figure 3.16 – Nur1 cannot rescue defective MEN pathway activation**
Nur1 deletion does not reduce the requirement for the MEN. Strains of the indicated genotypes were streaked on YPD agar plates and grown at the temperatures shown.

## 3.8 Nur1 may contribute to Cdc14 sequestration by influencing Cdk phosphorylation of Net1

### 3.8.1 Nur1 and Net1 physically interact

In order to gain more insight into the mechanism by which Nur1 prevents Cdc14 release, we examined whether Nur1 physically interacts with proteins involved in the control of mitotic exit. In a previous study, it was found that affinity purified fractions of Nur1, analysed by mass spectrometry, contained Heh1 and mitotic monopolin, as well as a Net1 peptide (Mekhail et al., 2008).

To confirm the physical interaction between Net1 and Nur1, a co-immunoprecipitation experiment was carried out. In strains in which Net1 is tagged with myc and Nur1 with Pk, we synchronised cells in G1 and released them to go synchronously through the cell cycle. At 15-minute intervals, the interaction of Net1 with Nur1 was monitored by immunoprecipitating Net1 from cell extracts using an α-myc antibody. The proteins co-immunoprecipitated, with little variation in interaction strength between different cell cycle stages (Fig. 3.17). In addition, the strain contained Cdc14 tagged with HA; Cdc14 was also detected to be co-immunoprecipitating with Net1 and Nur1 in our extracts (Fig. 3.17). While it is currently unknown with which of Net1 and/or Cdc14 Nur1 makes direct contact, this finding opens the possibility that Nur1 directly influences Cdc14 inhibition together with Net1.

### 3.8.2 Nur1's effect on Cdc14 is dependent on Net1's phosphorylation status

One of the key events during Cdc14 activation early in anaphase is Cdk phosphorylation of Net1 on at least six Cdk consensus phosphorylation sites. One hypothesis for the mechanistic role of Nur1 in Cdc14 release is therefore that Nur1 opposes Cdc14 release by influencing the phosphorylation state of Net1. In order to start investigating this, I took advantage of the *net1-6Cdk* allele that lacks these six Cdk phosphorylation sites and, as a consequence, delays activation of Cdc14 and causes a small delay in mitotic exit. If Nur1's role in Cdc14 activation is mediated by a

counteracting effect on Net1 phosphorylation, then Nur1 inactivation will not be able to rescue the mitotic exit delay of *net1-6Cdk* cells. On the other hand, if Nur1 is acting to inhibit Cdc14 in a pathway different from influencing Net1 phosphorylation, then its deletion should be able to advance Cdc14 activation in *net1-6Cdk* cells, in a similar way to what was observed in the *spo12*Δ background. However Nur1 deletion did not advance the timing of Cdc14 release or reduce the delay in mitotic exit of *net1-6Cdk* cells (Fig. 3.18). This suggests that Nur1 contributes to Cdc14 regulation most likely by influencing Cdk phosphorylation of Net1.

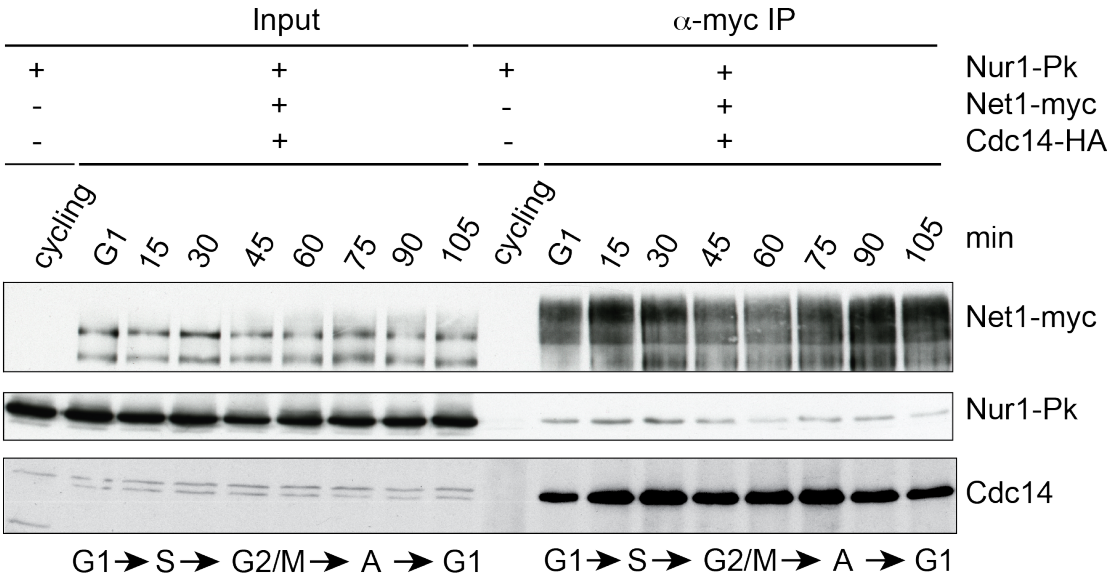



**Figure 3.17 – Nur1, Net1 and Cdc14 form a complex**
Nur1 interacts with Net1 throughout the cell cycle. Cell extracts were prepared from aliquots of a culture passing through a synchronous cell cycle at the indicated times. Co-immunoprecipitation of Nur1-Pk with Net1-myc was examined. A strain expressing Nur1-Pk but lacking the Net1-myc epitope served as a control. Cell cycle progression was monitored by FACS analysis of DNA content; the prevalent cell cycle stages are indicated.

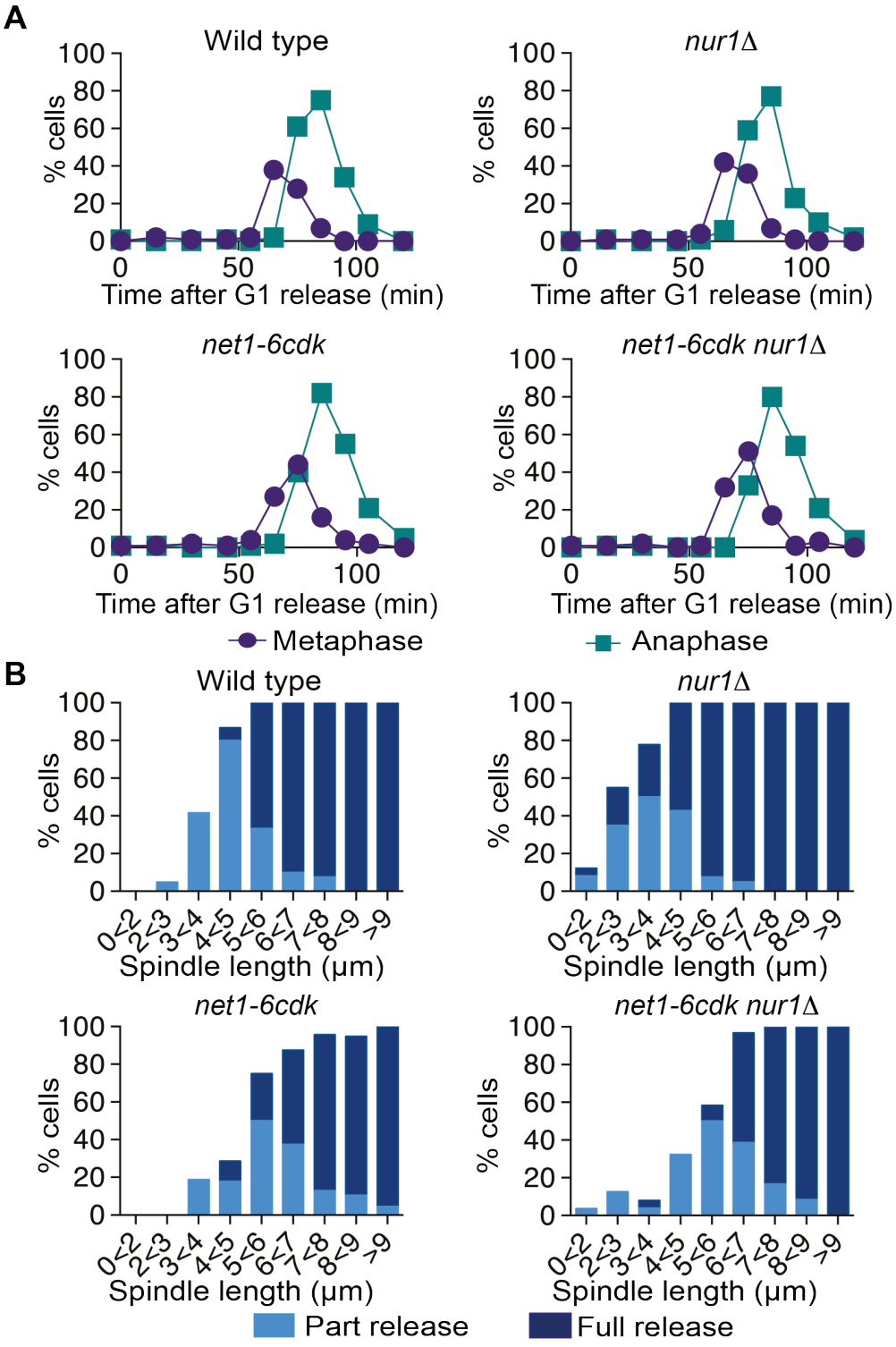



**Figure 3.18 – Nur1's effect on Cdc14 is dependent on Net1's phosphorylation status**
**A.** Progression through mitosis of wild type, *nur1Δ*, *net1-6cdk* and *nur1Δ net1-6cdk* cells was measured by counting percentages of cells displaying metaphase (1–3 µm) or anaphase (>3 µm) spindles during synchronous cell cycle progression following α-factor arrest and release. **B.** Quantification of Cdc14 release versus spindle length, during the experiment above.

## 3.9 Discussion and conclusions

### 3.9.1 Nur1, a novel player in budding yeast mitotic exit

The paramount importance of Cdc14 in budding yeast mitotic exit has become evident, with the list of its targets and functions ever increasing. For instance, its incompletely understood role in cytokinesis has recently come into focus, with a range of Cdc14 targets identified whose dephosphorylation is necessary for completion of this cell cycle stage (Kao et al., 2014; Kuilman et al., 2015). Concomitantly, our understanding of the regulation of the release and activity of this phosphatase is also deepening.

At the beginning of this thesis, I set out to further our knowledge of the role of Cdc14 in rDNA condensation, resolution and segregation. A promising target was recognised in the guise of the nuclear rim protein Nur1 – both identified as a Cdc14 target in a mass spectrometry screen and with a known role in regulating rDNA repeats. After confirming, both *in vivo* and *in vitro*, that the protein was a *bona fide* Cdc14 target, dephosphorylated by mid-anaphase, I created a version of Nur1 that, due to being fused to the cyclin Clb2, could no longer be dephosphorylated. This fusion led to a reduction in viability, as well as a noticeable delay in rDNA segregation, with the persistence of anaphase bridges formed by the rDNA repeats – characteristic of mis-segregation and chromosomal instability.

However, upon further investigation, it was revealed that the delay in rDNA segregation was in fact due to a higher-level delay in mitotic exit itself. As such, I found that fusion of Nur1 to Clb2 caused a delay in Cdc14 release, which was responsible for persistence of anaphase spindles, delayed destruction of Clb2 and delayed dephosphorylation of Orc6, as well as a lag in accumulation of Sic1. Intriguingly, it also resulted in a delay in the metaphase to anaphase transition as measured by Pds1 destruction and ratio of metaphase/anaphase spindles in synchronous cell cycles. It could be that a small amount of Cdc14 needs to be released late in metaphase or early in anaphase. Indeed, a role for active Cdc14 in metaphase has been proposed. It has been shown to be involved in promoting the dephosphorylation of the kinetochore protein Dsn1, possibly promoting chromosome biorientation (Akiyoshi and Biggins, 2010).

Further, a function for Cdc14 in keeping Spo12 dephosphorylated and inactive until early anaphase has also been proposed (Tomson et al., 2009). Alternatively, Nur1 may have functions in metaphase other than regulating Cdc14 release – this could be a subject for further studies.

### 3.9.2 A relationship between Nur1's role in mitotic exit and its established role in maintenance of genome stability?

I have established that Nur1 plays an important role in in promoting accurate timing of mitotic exit events through regulating Cdc14. However this protein was previously characterised as necessary for the maintenance of genome stability and replicative life span, important for physically linking the genome to the nuclear membrane – giving it an identity as a CLIP (chromosome linkage INM proteins). Are these roles independent functions for this protein, or somehow related?

Interestingly, I found that Nur1-Clb2 fusion increased Unequal Sister Chromatid Exchange – thereby causing unequal segregation of the rDNA repeats. Given that it was previously reported that *nur1Δ* also causes an increase in Unequal Sister Chromatid Exchange as well as changes in rDNA copy number, it will be interesting to examine whether Nur1's role in tethering the rDNA to the nuclear envelope is somehow dependent on its phosphorylation state. It is unknown whether or how the tethering of the rDNA changes during the cell cycle, for instance if the tethering is modified or maintained during condensation and segregation, or how the rDNA moves relative to the nuclear envelope. In fact, Nur1 was previously predicted to be a Cdk-regulated nucleocytoplasmic shuttling protein, due to the Cdk site within its putative nuclear localisation signal (Kosugi, Hasebe, Tomita and Yanagawa, 2009). This indicates that its localisation may well be modified in a cell cycle dependent manner, having possible implications both for Cdc14 release and rDNA tethering.

Having performed a superficial examination of Nur1 localisation throughout the cell cycle by immunofluorescence analysis, this was not something I observed. A slight enrichment of the immunofluorescence signal at the nuclear envelope was noticed, whatever the cell cycle stage, befitting the previously annotated localisation of the

protein and its interaction with nucleolar proteins throughout the cell cycle. However background signal was very high, so cell cycle dependent modifications in localisation cannot be ruled out.

Related to this, one of the phenotypes reported in *net1-1* mutants was a high rate of chromosome loss (Shou and Deshaies, 2002) indicating that proteins with a role in Cdc14 regulation might more generally play a role in accurate chromosome segregation.

### 3.9.3 A dual role for Nur1 in Cdc14 release

Both deleting Nur1, and making a *nur1(9A)* mutant, caused Cdc14 to be released both earlier and, in the case of the Nur1 deletion, more abruptly than in wild type cells. Indeed, in a small proportion of *nur1Δ* cells Cdc14 was released both into the nucleus and cytoplasm in cells with a cytological appearance typical of metaphase rather than anaphase, when the spindles had formed but not yet properly started elongating. This could indicate that the primary function of phosphorylated Nur1 is to prevent Cdc14 from being released too early, or too much Cdc14 from being released in the early stages of anaphase. This could be rationalised with reference to a quantitative model of mitotic exit, in which Cdc14 substrates are thought to be dephosphorylated at a specific level of phosphatase activity versus Cdk kinase activity. Thus in very early anaphase, phospho-Nur1 makes sure that the levels of Cdc14 are kept low so that it only targets the very best substrates.

The fact that Nur1 deletion or *nur1(9A)* mutation partly rescue FEAR, but not MEN pathway mutants support a role for the dephosphorylation of this protein in early anaphase in order to aid Cdc14 release.

Firstly, upon FEAR pathway activation, a small amount of Cdc14 can be released, leading to a low level of active Cdc14 in the cell – enough to dephosphorylate the phosphatase's "early targets". Following this, Cdc14 can then promote an increase in its own activity levels by dephosphorylating Nur1, relieving this second level of inhibition. This process would be facilitated due to the concomitant drop in Cdk activity occurring as the cells progress through anaphase. This would cause release of higher amounts of

the phosphatase, with activity levels necessary for the dephosphorylation of the next round of targets. Finally, through the action of this positive feedback loop involving Nur1 dephosphorylation, enough Cdc14 would be released for the MEN pathway to be activated (Fig. 3.19).

Whether the dephosphorylation of Nur1 actually stimulates release of Cdc14, or just stops Nur1 from inhibiting it, is still an open question. Although I have shown that Nur1 interacts with Net1 throughout the cell cycle, the molecular mechanism by which phosphorylated Nur1 inhibits Cdc14 release is unclear. Indeed, the molecular mechanisms by which the FEAR and MEN pathways cause Cdc14 to be released from Net1, or indeed how Net1 sequesters Cdc14, also remain unknown. It is possible that Nur1 is influencing the interaction between Net1 and Cdc14, causing Net1 to have a tighter hold, or perhaps Nur1 itself contributes to sequestering the phosphatase. Upon dephosphorylation, the interaction would then be modified.

Thus it seems likely that Nur1 has a dual role in Cdc14 release, both preventing it when phosphorylated and stimulating it when dephosphorylated. In fact, there are several other proteins that may have similar dual roles towards Cdc14. For instance it has been shown that Tof2 restrains Cdc14 release early in anaphase (when the FEAR pathway is active), and activates it late in anaphase (when the MEN pathway is active) (Waples et al., 2009). Similarly, it is thought that Slk19, although activating Cdc14 release as part of the FEAR pathway actually prevents exit of Cdc14 from the nucleus, via a separate mechanism, until the MEN is active (Faust et al., 2013). Finally, this is not the only example of Cdc14 regulating its own release – as mentioned previously, it is thought that the phosphorylation state of Spo12 influences its FEAR pathway role, with Cdc14 maintaining it in a dephosphorylated and inactive state until early anaphase, when it is activated by Cdk phosphorylation (Tomson et al., 2009).

A robust positive feedback loop, in which Nur1 prevents Cdc14 from being released until dephosphorylated, but stimulates Cdc14 release upon this event, could explain how Cdc14 release is maintained between its initial activation by the FEAR pathway

and the sustained release caused by the MEN pathway. In this fashion, Nur1 could form a bridge between the two characterised Cdc14 release pathways (Fig. 3.19).

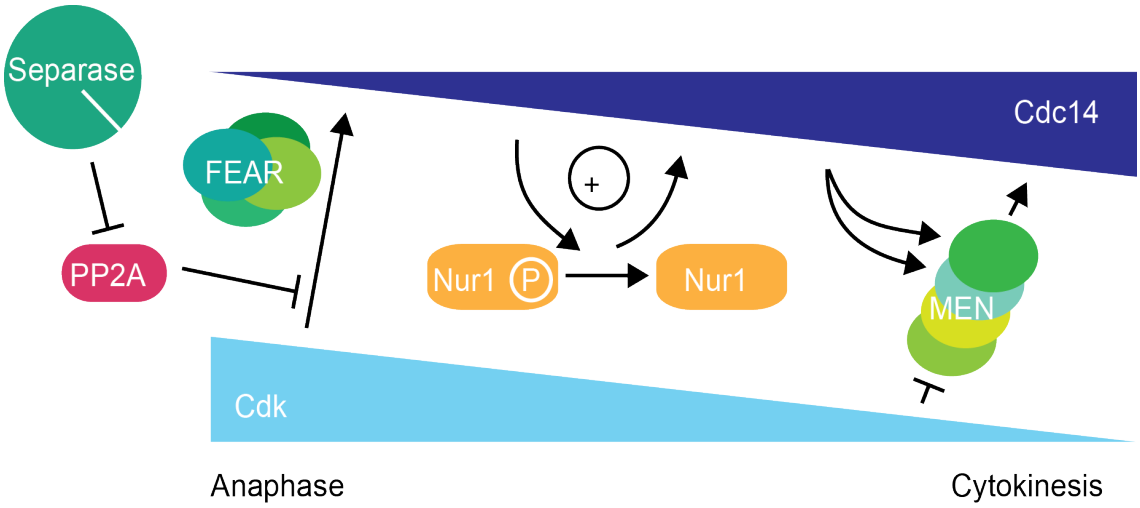



**Figure 3.19 – Nur1 establishes a positive feedback loop to promote Cdc14 release in early anaphase.**
At the metaphase to anaphase transition, Cdc14 starts to be released following APC-mediated securin destruction, which liberates separase to downregulate $PP2A^{Cdc55}$. This allows Cdk to phosphorylate Net1 to initiate Cdc14 release. At the same time Cdk activity is in decline, thus removing the original impetus for Cdc14 release. To counteract this, Nur1 is dephosphorylated by early released Cdc14. This turns off Nur1's ability to inhibit Cdc14 and thus helps to sustain and augment Cdc14 release until the MEN pathway becomes active to maintain Cdc14 release while Cdk activity is further downregulated.

### 3.9.4 Future perspectives

On the on one hand, there is a requirement to deepen our understanding of the role of Nur1, and Net1, beyond mitotic exit, notably in regulating the accuracy of chromosome partitioning. Further, the targets of Cdc14 necessary for the accurate condensation and segregation of the rDNA still remain to be identified.

In fact, how the genome, a spatially constricted entity, tethered to the nuclear envelope, organised into higher order structures, with a large amount of proteins bound to it, manages to first replicate, then condense, and finally accurately segregate to opposite parts of the cell is still incompletely understood, as is how all of this is regulated in a cell cycle dependent manner. As such, the relationships between the RENT complex and

Nur1 phosphorylation, and chromosome segregation and genome organisation, remain to be examined.

On the other hand, although the genetics behind the regulation of Cdc14 release are well-understood, the molecular mechanisms behind this are still unclear. As such, the next step in understanding how this process works is to work out, mechanistically, how phosphorylation of Net1 by the FEAR and MEN promotes Cdc14 release, and further, how Nur1 contributes to this process.

In this chapter, I have focused on the role of phosphatases in budding yeast mitotic exit, specifically giving new insights into the regulation of Cdc14 activity in this cell cycle phase. The process of mitotic exit is already fairly well understood, and the importance of phosphatase activity, and the regulation of this activity have been established.

However, what is less well understood is the role of Cdk-opposing phosphatases in other cell cycle phases. In particular, whether such phosphatases have a role in controlling the ordering of Cdk substrate phosphorylation in interphase, and by doing so the ordering of cell cycle events such as S phase or G2, is unknown.

# Chapter 4. Results: A role for Cdk-opposing phosphatases in budding yeast interphase

As discussed in Chapter 1, there are three main Cdk-opposing phosphatases in budding yeast: Cdc14, PP1 and PP2A, the roles of the latter two being diversified by association with a variety of regulatory subunits. Extrapolating from the established role of Cdc14 in controlling the ordered dephosphorylation of Cdk substrates in a quantitative manner during budding yeast mitotic, it can be postulated that in a similar manner, Cdk-opposing phosphatase activity could be controlling ordered Cdk substrate phosphorylation during interphase. As during mitotic exit, we can hypothesise that the timing of substrate phosphorylation during interphase would be controlled by the ratio of Cdk activity to Cdk-opposing phosphatase activity. Therefore the combination of rising Cdk activity, along with constant (or declining) Cdk-opposing phosphatase activity, would determine the order in which substrates are phosphorylated. Whether any, all, or none of these three phosphatases are indeed playing such a role was the second question I aimed to address.

## 4.1 Strategic approach for examining the importance of phosphatase activity in interphase

In order to look at the role of phosphatases in interphase, my first approach was to remove phosphatase activity, and the second to have a biochemical and/or phenotypic read-out for the consequences of this removal.

### 4.1.1 Removing phosphatase activity

Cdc14 is a single-subunit phosphatase, thus removing its activity can simply rely on the use of temperature-sensitive yeast strains, in which Cdc14 is active at the permissive temperature of 25°C and inactivated at the restrictive temperature of 35.5°C. Similarly, although PP1 is a multi-subunit phosphatase, it only has one catalytic subunit, Glc7, which can also be inactivated through the use of temperature sensitive alleles.

However, creating a strain lacking PP2A activity is more challenging. Firstly, this protein is a multi-subunit phosphatase, composed of a scaffold subunit, a catalytic subunit and a regulatory subunit. Moreover, the *S. cerevisiae* genome encodes two near-identical catalytic subunits, Pph21 and Pph22, each of which provides about 50% of the PP2A activity in the cell, and either of which can compensate for deletion of the other (Jiang, 2006; Sneddon, Cohen and Stark, 1990). In fact, even deletion of both subunits together does not completely abrogate PP2A activity, as the PP2A-like phosphatase Pph3, which is structurally similar to Pph21 and Pph22, is thought to be able to partly compensate for their absence (Jiang, 2006). In order to completely remove PP2A catalytic activity it is therefore necessary to use a strain in which both Pph22 and Pph3 are deleted, combined with a temperature sensitive allele of Pph21. However, cell viability and growth is severely compromised in this strain, and in fact these cells were unable to release from a G1, α-factor induced, arrest, at restrictive temperature.

Given these limitations, I decided to focus on the role of $PP2A^{Cdc55}$. As discussed in Chapter 1, this version of the phosphatase is known to play a variety of roles in the *S. cerevisiae* cell cycle. Further, it is highly active in interphase, and in other eukaryotic systems, its homologs have been shown to play a negative role in mitotic entry. Further, a role for $PP2A^{Rts1}$ has so far only been confirmed in very early interphase, and in cell size regulation. We can therefore postulate that $PP2A^{Cdc55}$ is the version of this phosphatase most likely to be implicated in cell cycle progression.

Deletion of Cdc55 is not lethal, however causes severe growth defects, due to its role in opposing Swe1 activity in interphase. As discussed in Chapter 1, this is due to the fact that Cdc55 is required for timely progression through the G2/M transition. In fact, as I was interested in understanding roles of PP2A in interphase other than its previously established role at the G2/M transition, I created a double deletion strain lacking both Cdc55 and Swe1, which restored cell viability and virtually normal cell cycle progression, as previously reported (Harvey et al., 2011; Yang, Jiang, Gentry and Hallberg, 2000). As such, *swe1Δ* and *swe1Δ cdc55Δ* cells release from an α-factor induced G1 arrest and carry out DNA replication with identical timing, as monitored by FACS analysis of DNA content (Fig. 4.1A). Further, they enter metaphase and then

anaphase together, as monitored by counting metaphase spindles (1–3 μm) and anaphase spindles (>3 μm) as cells progressed through the cell cycle, although *swe1Δ cdc55Δ* cells then have a slight delay in progression through anaphase (Fig. 4.1B). Therefore using a *swe1Δ cdc55Δ* strain, with a *swe1Δ* strain as a control, seems like an appropriate model to study the role of $PP2A^{Cdc55}$ in interphase.

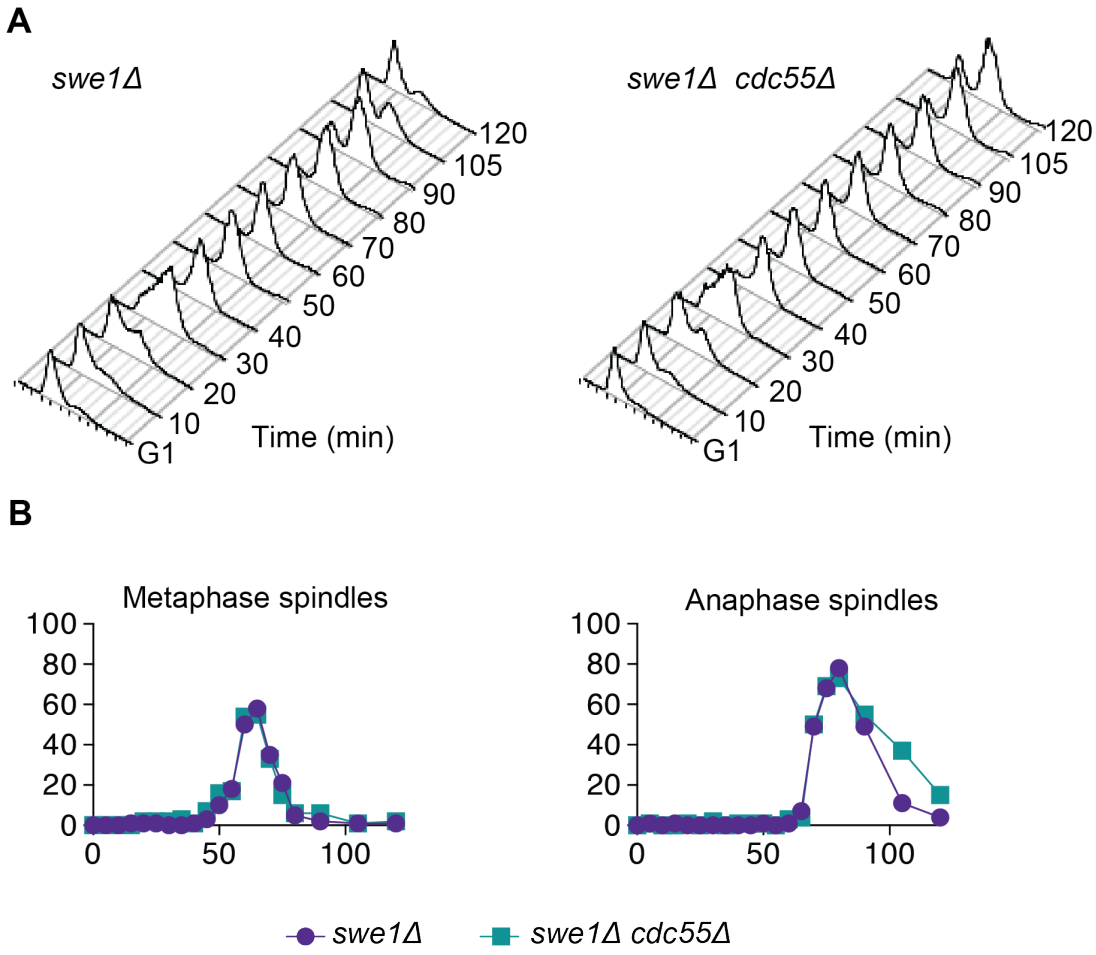



**Figure 4.1 – *swe1Δ* and *swe1Δ cdc55Δ* strains progress through interphase and enter mitosis with identical timings**
Cell cycle progression of *swe1Δ* and *swe1Δ cdc55Δ* cells was monitored **A.** by FACS analysis of DNA content, and **B.** by counting percentages of cells displaying metaphase (1–3 μm) or anaphase (>3 μm) spindles during synchronous cell cycle progression following α-factor arrest and release.

### 4.1.2 Measuring changes in Cdk phosphorylation levels

Once phosphatase activity has been removed, there arises a need to directly measure what happens to Cdk substrate phosphorylation in interphase. Cells can be synchronised in G1 by α-factor treatment, released to progress through the cell cycle synchronously and protein extracts prepared at intervals. Using these extracts, global levels of Cdk phosphorylation can be monitored by carrying out a Western blot using a range of antibodies against phosphorylated Cdk sites, which recognise phosphorylation of numerous Cdk phosphorylation sites. In addition, by tagging specific proteins with an epitope tag, these proteins being usually phosphorylated in interphase, changes in their timing of phosphorylation may be observed by monitoring mobility shifts on an SDS-page gel.

## 4.2 Identification of interphase Cdk targets

### 4.2.1 A SILAC screen to identify G2/M Cdk targets

Initially, I hypothesised that Cdk-phosphatase activity would be most important at the later stages of interphase, and more specifically in G2, where Cdk activity is already high but cells have not yet formed spindles. In order to identify Cdk targets that are phosphorylated in metaphase but not in G2, I decided to perform a SILAC (Stable Isotope Labelling in Culture) screen, in which I compared G2 Cdk phosphorylation to Metaphase Cdk phosphorylation (Gruhler et al., 2005). Using this technique, in which a culture grown in the presence of heavy isotopes of lysine and arginine and a culture grown in the presence of light isotopes of arginine and lysine were combined, I was able to directly compare phosphorylated peptides between G2 and metaphase.

In order to compare phosphorylation in a "natural" cell cycle (i.e. without arresting cells with Nocodazole, for instance, which leads to artificially high levels of Cdk phosphorylation), cells (grown in either "light" or "heavy" media) were synchronised in G1 by α-factor pheromone arrest, released to progress synchronously through the cell cycle and samples taken at the time when the majority of the cells pass through G2 and M phase. To determine when these phases are happening, three repeats of this arrest

and release experiment were carried out before performing the SILAC experiment itself. G2 was taken to be after cells had duplicated their DNA (by measuring DNA content by FACS analysis), and 5 minutes before the start of spindle formation, and metaphase when metaphase spindles reached their peak of 70-80%, which was exactly 20 minutes after that (Fig. 4.2A-B).

For the SILAC experiment itself, both cultures (heavy and light) were synchronized in G1 using α-factor, at a 20-minute interval, and released, at a 20-minute interval, 150 minutes after the arrest (therefore, respectively, 150 minutes, and 170 minutes after the beginning of the experiment) (Fig. 4.2C). The cells were harvested together 45 minutes after the release of the second culture (therefore, 65 minutes after the release of the first culture) at which point identical amounts of cells from the light and heavy cultures were combined, and processed for mass spectrometry analysis. The experiment was repeated twice, once with the heavy culture in G2 and light in M, and the second time vice-versa. An asynchronous control was also harvested.

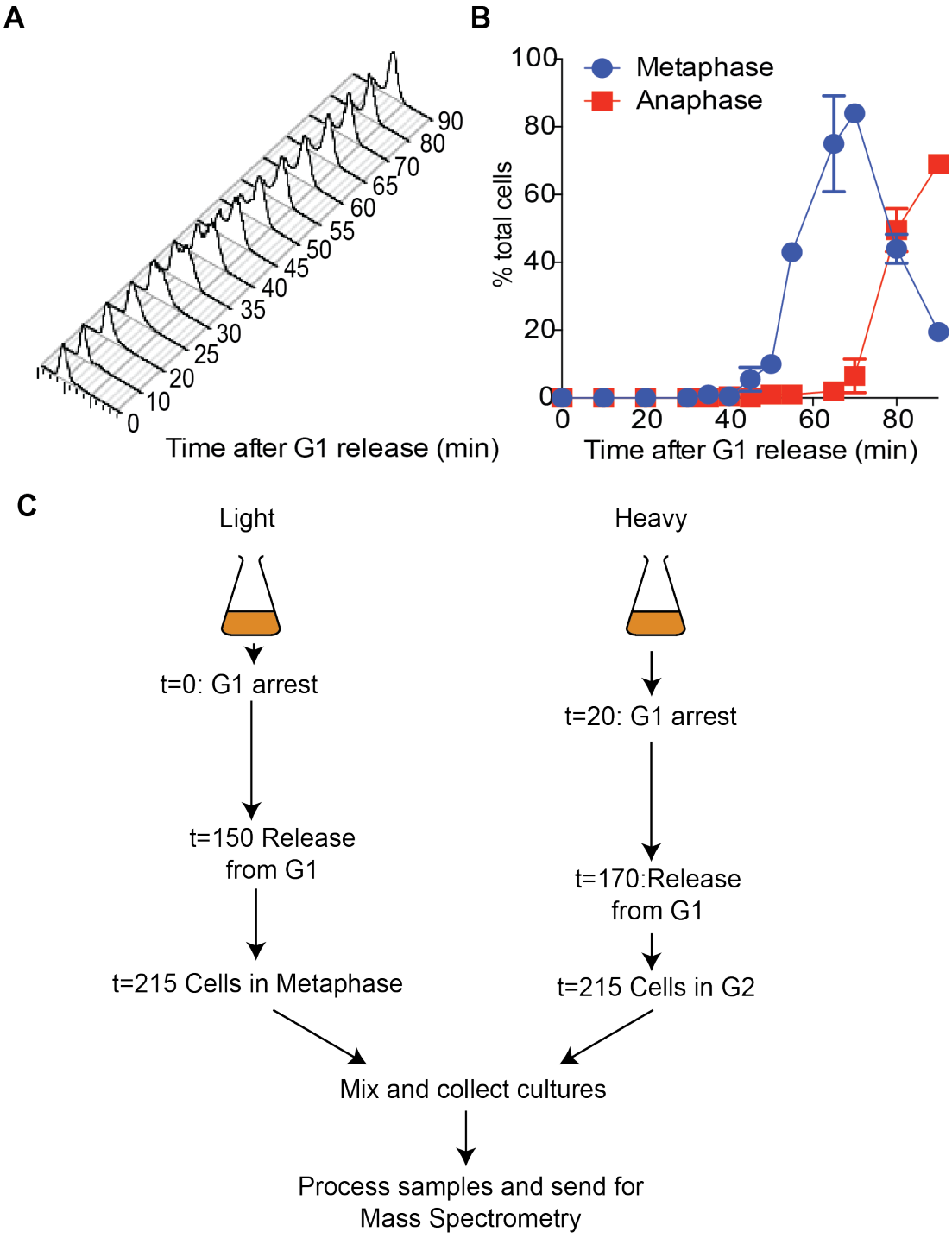
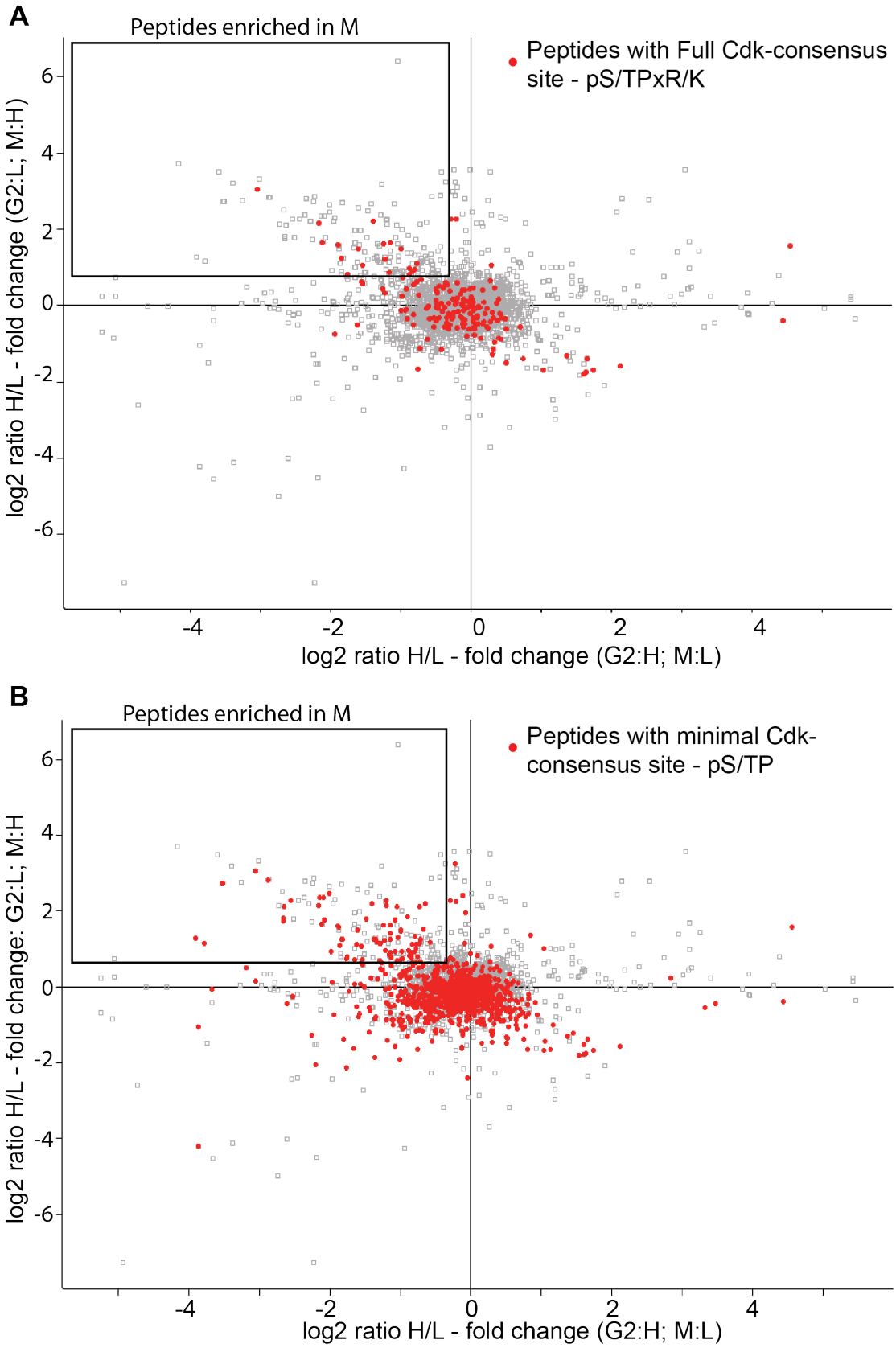



**Figure 4.2 – Design of a SILAC screen to compare G2 vs. M Cdk phosphorylation**
Cell cycle progression of a wild type strain in YNB media was monitored **A.** by FACS analysis of DNA content, and **B.** by counting percentages of cells displaying metaphase (1–3 μm) or anaphase (>3 μm) spindles during synchronous cell cycle progression following α-factor arrest and release. **C.** In order to perform a SILAC screen, cells were grown in media with either heavy or light Lys/Arg isotopes, arrested in G1 with a 20 minute interval and harvested together and combined, before being processed and sent for mass spectrometry analysis.

The “G2-Heavy/M-Light” and “G2-Light/M heavy” experiments were correlated, and only phosphorylated peptides found to be enriched in metaphase in both experiments were taken into account in the final analysis of the results. The peptides were plotted graphically, correlating the ratio of heavy versus light for each peptide identified, between the two experiments, giving rise to a scatter plot in which peptides enriched in metaphase can be visualised. In total, around 300 phosphopeptides were found to enriched in metaphase (Fig. 4.3). Of these around 100 had Cdk consensus sites, of which 30 were full S/TPxR/K Cdk consensus sites (Fig. 4.3).

These 100 or so peptides covered a variety of proteins, a handful of which were chosen for further study. Of the peptides identified, I chose ones that had been identified at least once in all three experiments, had been identified more than once in at least one experiment and were not enriched in heavy or light samples in the asynchronous control. Further, for proteins in which several peptides had been identified, I chose only to study those in which the majority of the identified phospho-peptides were enriched in metaphase. Proteins chosen for further study, and their previously known functions, are summed up in Table 4.1.


A
Peptides enriched in M
Peptides with Full Cdk-consensus site - pS/TPxR/K
log2 ratio H/L - fold change (G2:L; M:H)
log2 ratio H/L - fold change (G2:H; M:L)
B
Peptides enriched in M
Peptides with minimal Cdk-consensus site - pS/TP
log2 ratio H/L - fold change: G2:L; M:H
log2 ratio H/L - fold change (G2:H; M:L)

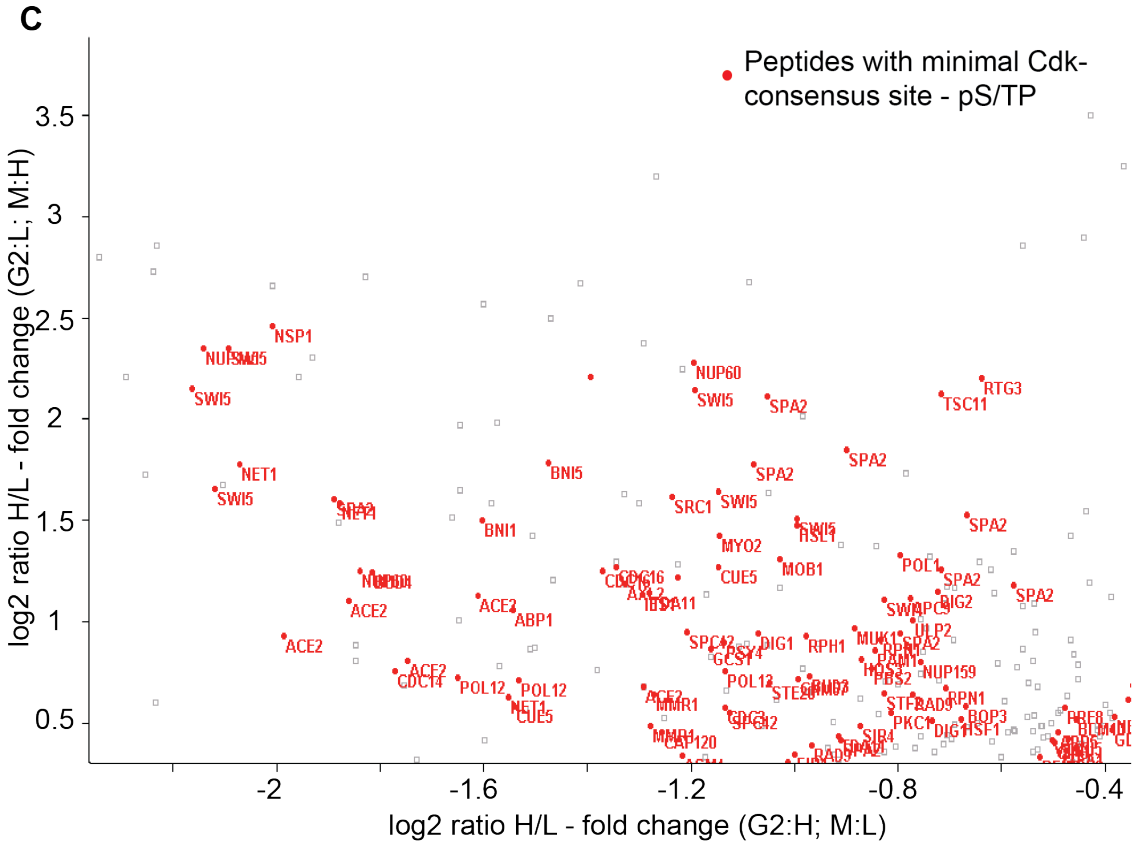



**Figure 4.3 – Metaphase Cdk targets identified by SILAC**
Two reverse SILAC experiments (heavy=G2 and light=M, and vice-versa) were correlated to determine which peptides were enriched in metaphase (rectangle in top-left quadrant (**A**-**B**). Cdk phosphorylated phosphopeptides were identified, on both full (**A**) and minimal Cdk sites, (**B**), and the identity of those enriched in metaphase was determined (**C**).

| Protein | Number of unique phosphopeptides (enriched in M) | Number of full CDK sites | Known CDK target | Function |
|---|---|---|---|---|
| **Spa2** | 8 | 2 | Yes | Polarised growth, cytokinesis |
| **Apc9** | 1 | 1 | Yes | APC complex |
| **Bik1** | 2 (same site) | 1 | No | Important for positioning of the spindle (+TIP protein) |
| **Bud4** | 3 | 0 | Yes | Cytokinesis |
| **Net1** | 7 | 1 | Yes | FEAR complex |
| **Src1** | 2 | 0 | Yes | Tethering of telomeres/rDNA to nuclear envelope and sister chromatid segregation |
| **Hsl1** | 1 | 1 | No | Cell cycle kinase, important for Swe1 degradation |

**Table 4.1 – Metaphase Cdk targets identified in the SILAC screen and their previously characterised roles**

I subsequently carried out cell cycle Western blot analysis on these proteins, to examine whether I could identify the timing of their phosphorylation by examining mobility shifts by SDS-Page electrophoresis. Cells were synchronised in G1 by α-factor treatment and released to proceed through the cell cycle synchronously. Unfortunately, for all of the above proteins, either a mobility shift was not identified, it did not vary in a cell cycle dependent manner, or the timing of phosphorylation was unclear due to the presence of too many bands.

### 4.2.2 Identifying Cdk targets – a candidate approach

This led me to a second, more biased, approach in order to identify interphase Cdk targets. I began a candidate approach, in which I searched through the literature for proteins that were known Cdk targets with known phospho-shifts, and phosphorylated with various timings in interphase of the cell cycle. A summary of the proteins chosen and their timing of phosphorylation is presented in Table 4.2.

| Protein | Phosphorylation timing |
|---|---|
| **Ndd1** | Phosphorylated in S phase, hyperphosphorylated in metaphase |
| **Sli15** | Slowly phosphorylated between S and G2 |
| **Ask1** | G1/S |
| **Swi5** | Metaphase (but transcriptionally regulated) |
| **Ase1** | Phosphorylated in S phase, hyperphosphorylated in metaphase |
| **Mms4** | Phosphorylated in metaphase |
| **Acm1** | G1/S - phosphorylation controls stability |

**Table 4.2 – Candidate interphase Cdk targets**
Candidate interphase Cdk targets were identified from searching the available literature and published data.

After having chosen these target proteins, I then proceeded to determine whether Cdk phosphorylation was altered in the absence of phosphatase activity, beginning with $PP2A^{Cdc55.}$

## 4.3 Removing PP2A$^{Cdc55}$ activity leads to advanced phosphorylation of Cdk targets throughout interphase

### 4.3.1 Advanced phosphorylation of Ndd1, a G2/M phase Cdk target

Ndd1, a transcription factor important for the transcription of the "Clb2 cluster" of genes, is phosphorylated in G2/M. This phosphorylation is needed for the association of the protein with its binding partner Fkh2, leading to binding to DNA and transcription of target genes (Reynolds et al., 2003; Darieva et al., 2003). Ndd1 is vital for mitosis, controlling the transcription of 30 or so genes including Clb2 itself, as well as many other proteins involved in mitotic progression and cytokinesis (Loy, Lydall and Surana, 1999; Pic-Taylor, Darieva, Morgan and Sharrocks, 2004).

Ndd1 phosphorylation was monitored by mobility shifts on a Western blot in a synchronous cell cycle experiment, comparing *swe1Δ* and *swe1Δ cdc55Δ* cells. Phosphorylation of Ndd1 began earlier and was more extensive in the absence of Cdc55. Lower mobility bands were observed approximately 30-40 minutes after release from G1 in the absence of Cdc55; when the cells were still in S phase according to FACS analysis of DNA content. In contrast, slower migrating bands only appeared 50 minutes after release when Cdc55 was present in the cells, corresponding to the G2/M transition according to previous experiments (Fig. 4.1), and after the completion of S phase, according to the FACS profile. The intensity of the slower migrating bands was also increased in the *swe1Δ cdc55Δ* background, indicating a higher proportion of the phosphorylated form of the protein as compared to *swe1Δ* alone (Fig. 4.4).

As mentioned above, Ndd1 is important for transcription of Clb2, which it does when phosphorylated by Cdk. This suggests that advanced Ndd1 phosphorylation could lead to earlier transcription of Clb2. Clb2 levels were viewed by western blot in the experiment in Figure 4.4, comparing *swe1Δ* and *swe1Δ cdc55Δ* in a synchronous cell cycle. No difference in timing of appearance of the protein was apparent between the two strains, although Clb2 persisted for longer in the absence of Cdc55 (Fig. 4.5).

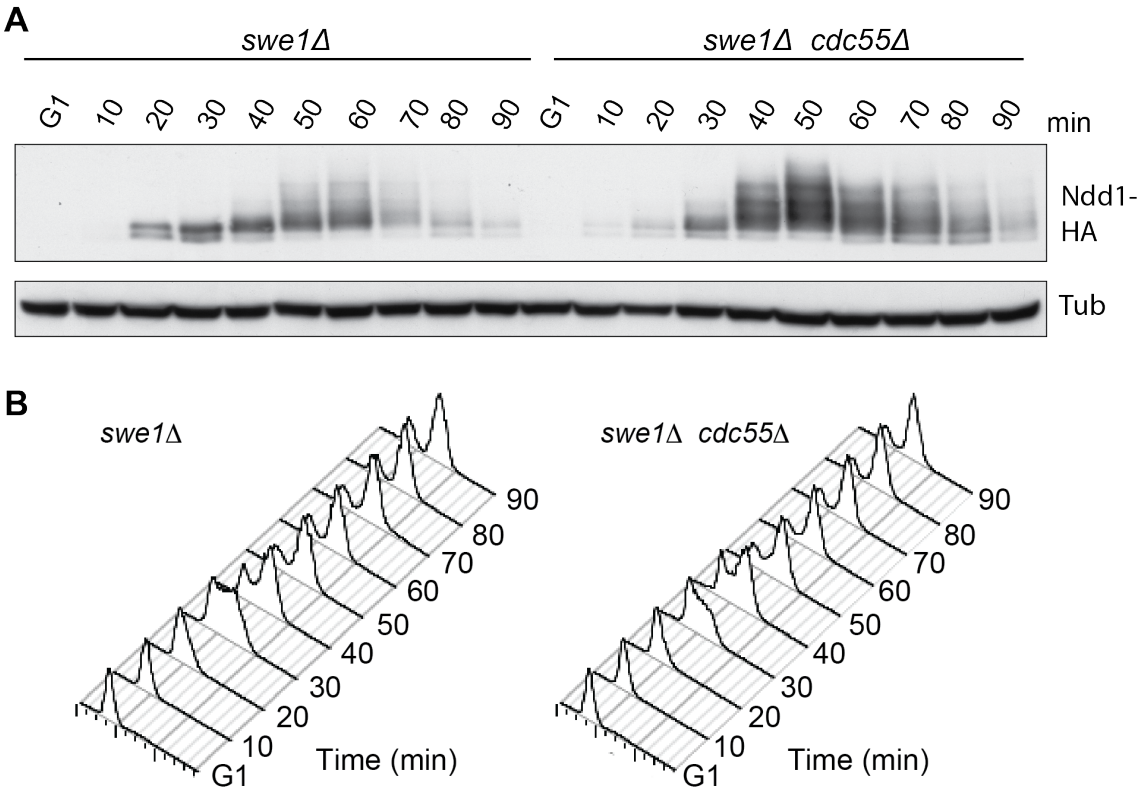



**Figure 4.4 – Phosphorylation of Ndd1 is advanced in the absence of Cdc55**
**A**. Ndd1 phosphorylation was monitored using an anti-HA antibody at regular intervals during synchronous cell cycle progression following α-factor arrest and release, in *swe1Δ* and *swe1Δ cdc55Δ* strains. Tubulin was used as a loading control **B**. Cell cycle progression was monitored by FACS analysis of DNA content.

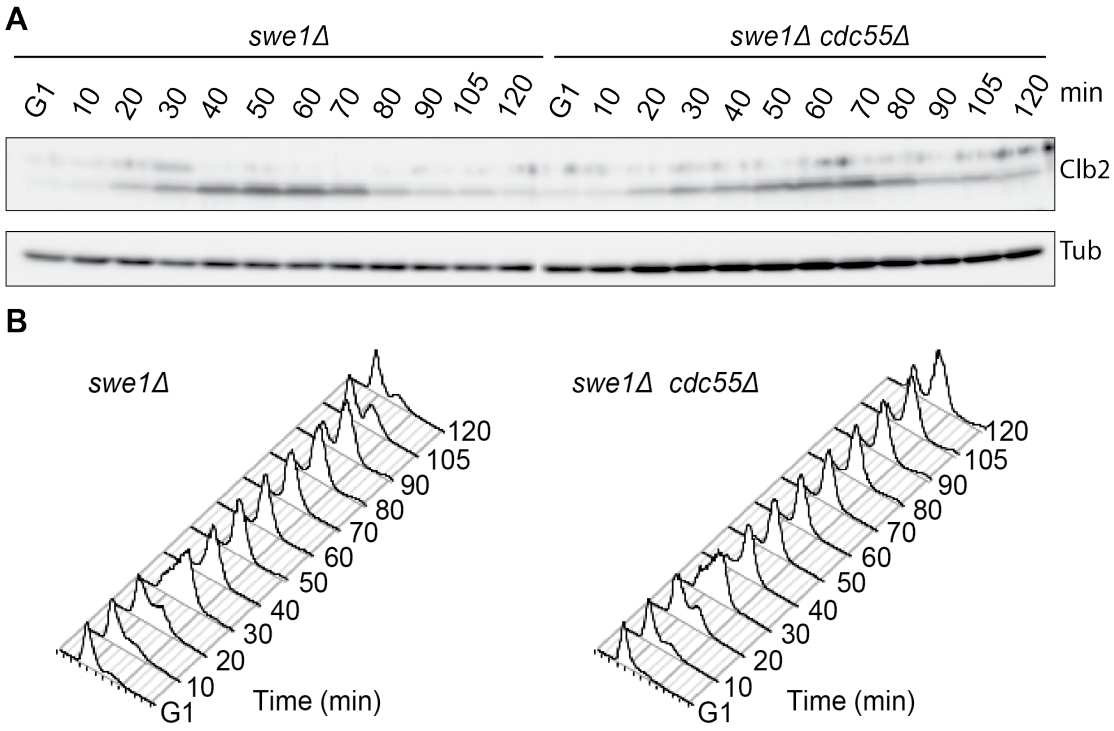



**Figure 4.5 – Clb2 is not expressed earlier in *swe1Δ cdc55Δ* than in *swe1Δ***
**A**. Clb2 levels were monitored using an anti-Clb2 antibody at regular intervals during synchronous cell cycle progression following α-factor arrest and release in *swe1Δ* and *swe1Δ cdc55Δ* strains. Tubulin was used as a loading control **B**. Cell cycle progression was monitored by FACS analysis of DNA content.

Phosphorylation of a G2/M phase Cdk target is advanced in the absence of Cdc55. Is the same true for proteins phosphorylated earlier in interphase?

### 4.3.2 Advanced phosphorylation of Sli15, an S/G2 phase Cdk target

The next Cdk target to be examined was Sli15, a member of the Chromosome Passenger Complex (CPC). In a normal cell cycle, Sli15 needs to be phosphorylated before metaphase to stop the CPC from binding to microtubules, and for the CPC to be retained at the centromere. It is usually phosphorylated by Cdk in late S phase, and can also be phosphorylated by Ipl1 (Widlund et al., 2006; Mirchenko and Uhlmann, 2010).

In similar synchronous cell cycle experiments as performed previously, it was observed that phosphorylation of Sli15, as monitored by mobility shifts on a Western blot, was advanced by around 10-15 minutes in *swe1Δ cdc55Δ* as compared to *swe1Δ*. As such, a slower migrating band was clearly already visible 10-20 minutes after release from G1 in the absence of Cdc55 (before S phase according to FACS analysis of the DNA content), whilst this was not the case until 20-30 minutes after release (during S phase) in the presence of Cdc55. However, in both cases, full phosphorylation of the protein – appearance of a second slower migrating band above the first – was not reached until 40-50 minutes after release from G1, after DNA replication was already completed (Fig. 4.6).

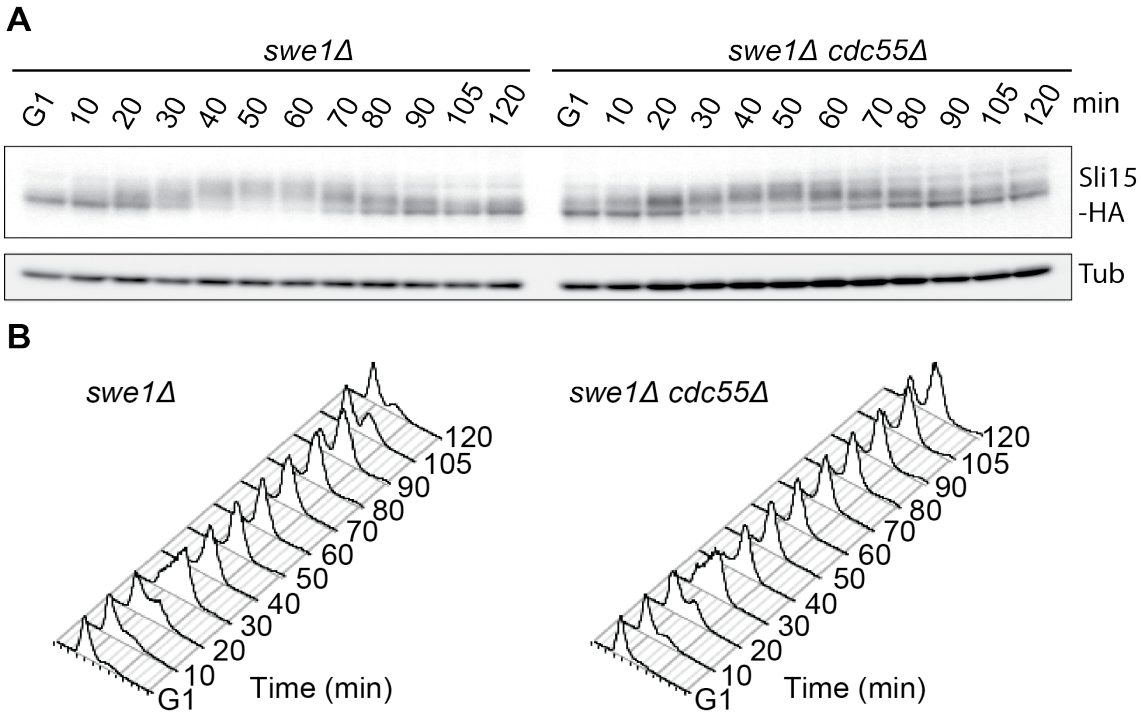



**Figure 4.6 – Phosphorylation of Sli15 is advanced in the absence of Cdc55**
**A**. Sli15 phosphorylation was monitored using an anti-HA antibody at regular intervals during synchronous cell cycle progression following α-factor arrest and release, in *swe1Δ* and *swe1Δ cdc55Δ* strains. Tubulin was used as a loading control **B**. Cell cycle progression was monitored by FACS analysis of DNA content.

### 4.3.3 Advanced phosphorylation of Acm1, a G1/S phase Cdk target

To check phosphorylation can also be advanced in very early interphase, for proteins phosphorylated by Cdk in complex with Cln-type cyclins rather than Clb-type Cyclins, the timing of phosphorylation of Acm1 was investigated. Acm1, a stoichiometric inhibitor of $APC^{Cdh1}$, is usually phosphorylated by Cdk in G1/S, which phosphorylation event directly controls the stability of the protein (Hall et al., 2008; Ostapenko, Burton, Wang and Solomon, 2008). Thus the abundance of the protein in interphase can be taken as an indirect readout for phosphorylation of the protein.

The abundance of Acm1 in interphase was quantified in a synchronous cell cycle experiment relating Acm1 levels to Tubulin levels by Western blot, again comparing *swe1Δ cdc55Δ* to *swe1Δ* cells (Fig. 4.7A-B). Acm1 band intensity was significantly

higher in early interphase (before DNA replication) in the absence of Cdc55, a strong indication that Acm1 must already be phosphorylated at this time (Fig. 4.7C).

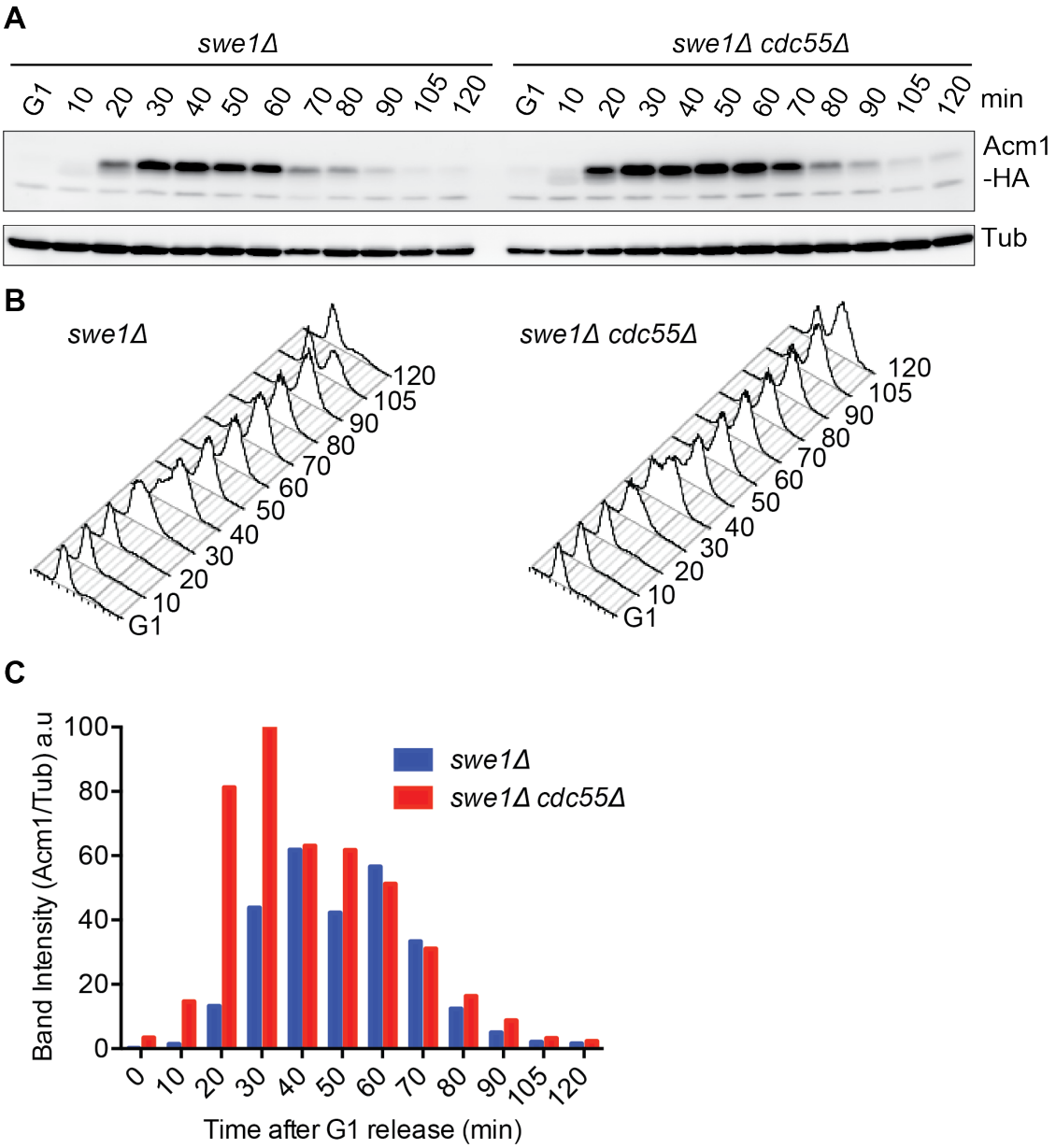



**Figure 4.7 – Phosphorylation of Acm1 is advanced in the absence of Cdc55**
**A.** Acm1 abundance was monitored using an anti-HA antibody at regular intervals during synchronous cell cycle progression following α-factor arrest and release, in *swe1Δ* and *swe1Δ cdc55Δ* strains. Tubulin was used as a loading control **B.** Cell cycle progression was monitored by FACS analysis of DNA content. **C.** Acm1 abundance was quantified at each time-point relative to the abundance of tubulin; pixel intensity was measured from ImageQuant images analysed in ImageJ, before signal saturation.

## 4.4 Removing PP2A$^{Cdc55}$ activity leads to a global increase in interphase Cdk phosphorylation

Given the identification of three specific Cdk targets whose phosphorylation was altered in the absence of PP2A$^{Cdc55}$, I examined whether global Cdk phosphorylation levels were affected by the absence of this phosphatase. *swe1Δ* and *swe1Δ cdc55Δ* cells were synchronised by α-factor treatment and released to proceed through the cell cycle (in two separate experiments) with protein extracts prepared at 10 minute intervals. These were run on SDS-Page gels, which were then probed with antibodies against phosphorylated full or minimal Cdk consensus sites; using anti-pSPxR/K, anti-pSP and anti-pTP antibodies.

Global Cdk phosphorylation, both on serines and on threonines, was increased throughout the cell cycle in the absence of Cdc55. The pattern of phosphorylation varied depending on the antibody used, however in all cases this increase was observed. Strikingly, the increase is observable even in the G1-arrested state, with several bands being more apparent in the absence of Cdc55, even this early in the cell cycle, with very low Cdk activity (Fig. 4.8, arrows). This is also true later in the cell cycle, as the cells progress through mitosis. Interestingly, the increase in levels of phosphorylation seems more dramatic when monitoring threonine, rather than serine, phosphorylation, however at this stage it is not clear whether this could be due to different antibody sensitivities/specificities.

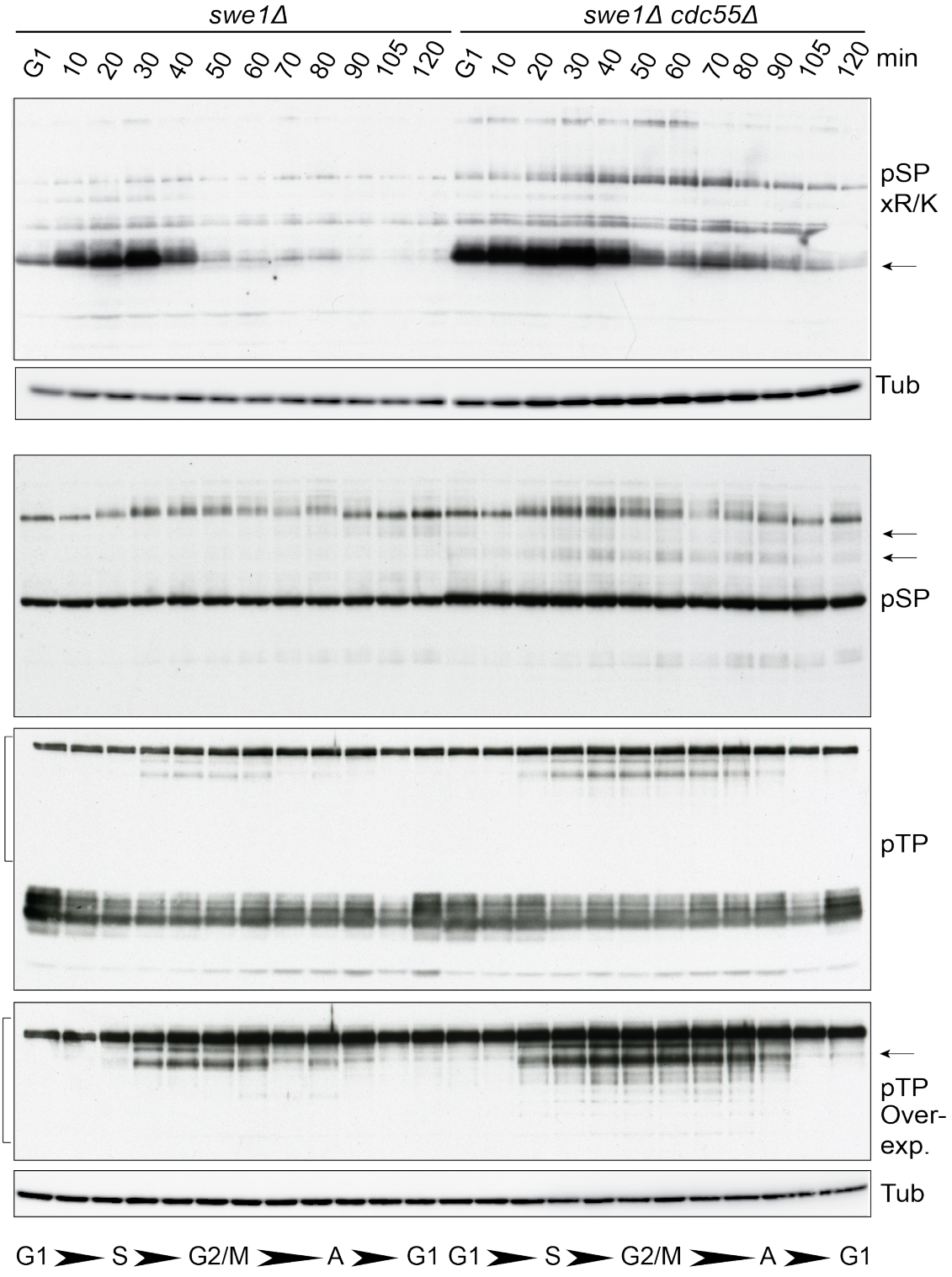



**Figure 4.8 – Global Cdk phosphorylation levels are increased in the absence of PP2A$^{Cdc55}$**

Cdk phosphorylation levels were monitored using various anti-phospho-Cdk sites antibodies at regular intervals during synchronous cell cycle progression following α-factor arrest and release. Tubulin was used as a loading control.

## 4.5 Absence of PP2A$^{Cdc55}$ is insufficient to allow entry into mitosis with reduced levels of Cdk activity

From the previous results, we can conclude that PP2A$^{Cdc55}$ regulates protein phosphorylation in interphase. Does this have any phenotypic consequences for cells?

In the first instance, it has already been noted that spindle formation does not occur any earlier in *swe1Δ cdc55Δ* than it does in *swe1Δ* cells alone, indicating that there seems to be no strong need for PP2A$^{Cdc55}$ in order to prevent too early mitotic entry (Fig. 4.1). However, this was in an unperturbed cell cycle, where Cdk activity is naturally high, and small differences in timing of spindle formation might be hard to spot. I therefore decided to examine whether in the presence of lower than usual Cdk activity, in a G2-arrested state, cells can proceed into metaphase if PP2A$^{Cdc55}$ activity is also removed (with the rationale that removing phosphatase activity would restore the correct kinase/phosphatase ratio for the G2/M transition to occur).

Two ways of arresting cells in G2 were tested. Firstly, using a *clb1Δ, clb3Δ, clb4Δ*, *clb2-ts* strain at restrictive temperature, where mitotic cyclins are absent, causing cells to arrest before mitotic entry (Amon, Tyers, Futcher and Nasmyth, 1993). Secondly, I used the Shokat allele of Cdc28 (*cdc28-as1*) in which Cdk activity can be modulated by adding rising concentrations of the inhibitor 1NM-PP1. Cells can be arrested either at the G1/S transition, with high concentrations of inhibitor, or at the G2/M transition, with low concentrations of inhibitor (Shokat et al., 2000).

In the first case, *clb1Δ clb3Δ clb4Δ clb2-ts* were combined with either *swe1Δ* or *swe1Δ* and *cdc55Δ*. Cells were transferred to restrictive temperature upon release from G1 and spindle formation and DNA replication was monitored. Interestingly, all *clb1Δ clb3Δ clb4Δ clb2-ts swe1Δ* cells escaped the arrest and formed spindles, however *clb1Δ clb3Δ clb4Δ clb2-ts swe1Δ cdc55Δ* cells did not – perhaps the cell cycle is too compromised in this situation.

Cells with *cdc28as-1* combined with either *swe1Δ* or *swe1Δ* and *cdc55Δ* were arrested either in G1 or G2 depending on the concentration of inhibitor used, and neither displayed any sign of spindle formation even 3 hours after release from G1 (Fig. 4.9).

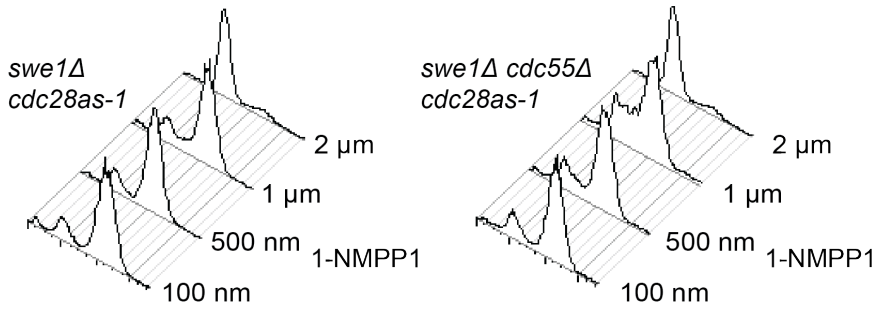



**Figure 4.9 – *cdc28as-1 swe1Δ* or *cdc28as-1 swe1Δ cdc55Δ* arrest at the same cell cycle stage**
Cell cycle stage was monitored by FACS analysis of DNA content in *cdc28as-1 swe1Δ* or *cdc28as-1 swe1Δ cdc55Δ* cells in the presence of concentrations of inhibitor 1-NmPP1 varying from 100 nm to 2 μm, 3 hours after release from an α-factor induced G1 arrest.

From these experiments, we can conclude that deletion of Cdc55 is insufficient to allow for mitotic entry with G2 levels of Cdk activity.

## 4.6 PP2A$^{Cdc55}$ preferentially opposes Cdk phosphorylation on threonines

### 4.6.1 A SILAC screen comparing interphase Cdk phosphorylation between *swe1Δ* and *swe1Δ cdc55Δ*

To further understand the role of PP2A$^{Cdc55}$ in opposing Cdk phosphorylation in interphase, from a more global point of view rather than focusing on specific Cdk targets, a second SILAC screen was carried out. In this screen, Cdk phosphorylation was compared between *swe1Δ* and *swe1Δ cdc55Δ* at different points in interphase. Cells of both genotypes were synchronised in G1 using α-factor. After the cells were determined to be fully arrested, the first SILAC sample was taken, mixing equal amounts of both cultures. The cultures were then released to progress through the cell cycle and two further samples were taken for SILAC analysis, respectively during mid-S phase, and G2. The timing of mid-S phase and G2 was determined in test experiments by monitoring DNA content by FACS analysis and formation of spindles by immunofluorescence, with S phase being when the DNA content was between 1C and 2C, and G2 when the DNA had fully replicated but the mitotic spindle had yet to form, as in the previous SILAC experiment. Each sample was confirmed to have been taken at the correct time (by the same measures) before samples were submitted for SILAC analysis. Each experiment was performed twice, reversing the "Heavy" and "Light" labelling between the *swe1Δ* and *swe1Δ cdc55Δ*.

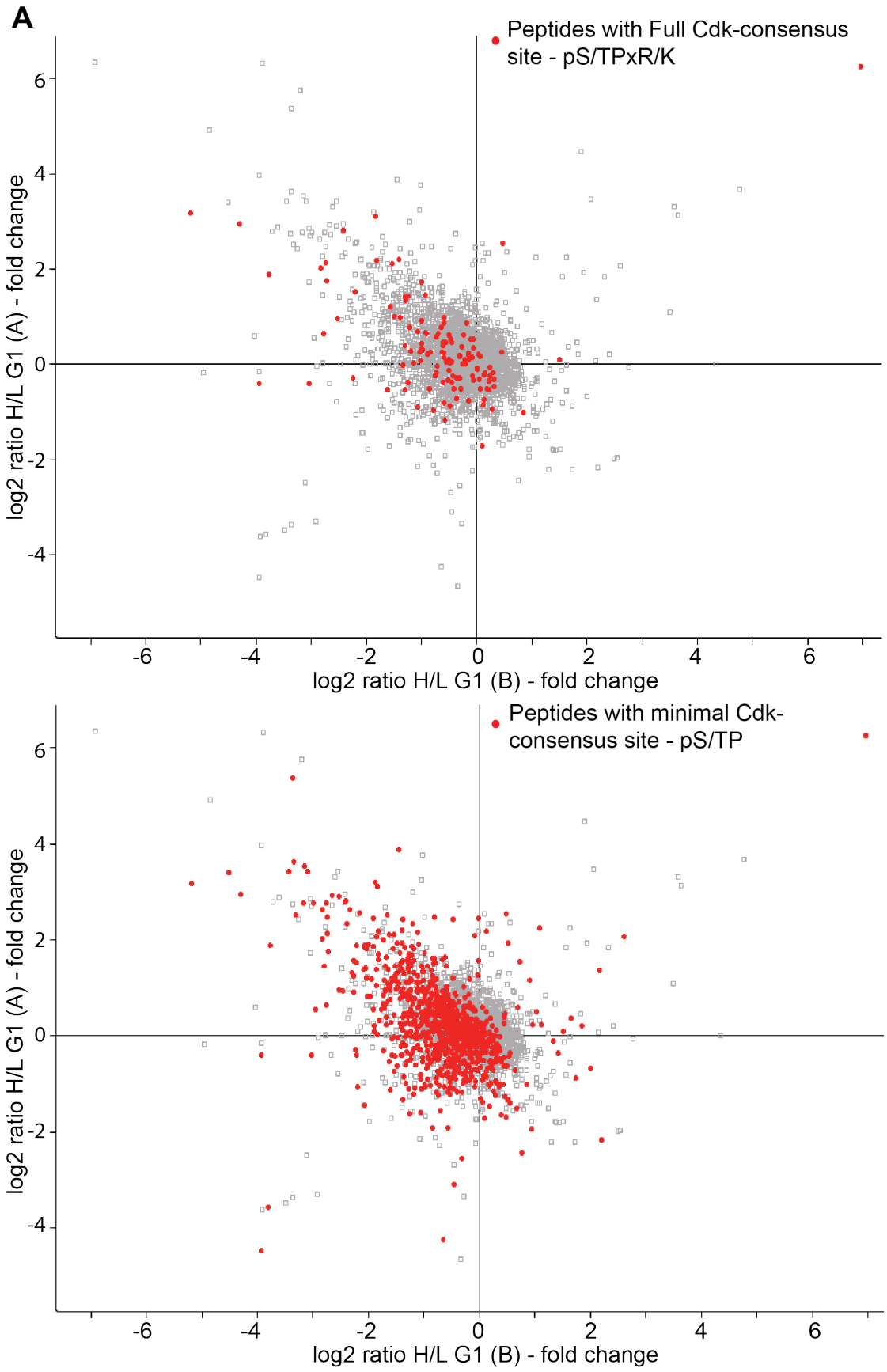
In the two repeats of the experiment, and over the three time-points, a total of 5307 phosphosites were identified, with 4589 being quantified, at a phosphopeptide enrichment efficiency of 66.6 %.

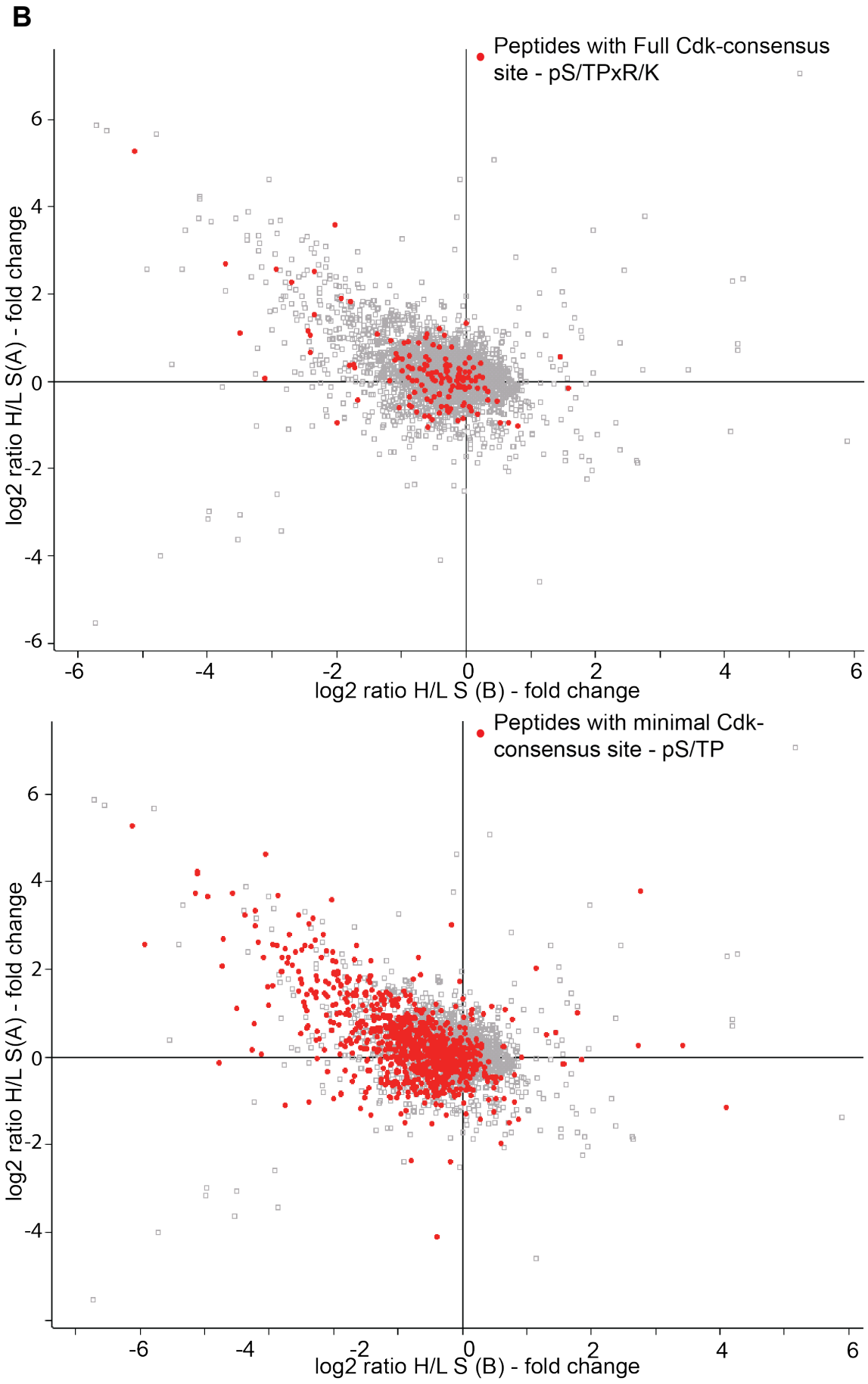
At each time point, relative phosphopeptide abundance (ratio of heavy/light versions of each identified phosphopeptide) was quantified. Over all of the sample mixes, it was found that around 12-20% of all phosphopeptides were enriched in the *swe1Δ cdc55Δ* sample compared to the *swe1Δ*, whether heavy or light, and, vice-versa, around 2.5-5%

of phosphopeptides were enriched in the *swe1Δ* sample as compared to the *swe1Δ cdc55Δ* sample.

The two repeats of the experiments were then correlated to provide further statistical significance in determining whether Cdk-motif containing phosphopeptides were enriched in either the presence or absence of phosphatase activity. Examining scatter plots of these correlations, it is immediately apparent that Cdk-containing phosphopeptides, both when looking at full Cdk consensus site motifs (pS/pTPxR/K) and minimal Cdk consensus site motifs (pS/sTP) appear to be enriched in the absence of $PP2A^{Cdc55}$ phosphatase activity, at all cell cycle stages (top-left quadrant of each scatter plot, Fig. 4.10). For further details and the results of each individual repeat (non-correlated) see Appendix 6.1 (Fig. 6.2).

In order to control for the quality and accuracy of the results, and to counter for any bias introduced by the labelling itself, control SILAC experiments were performed in which, at each time point (G1/S/G2), *swe1Δ* vs. *swe1Δ* (heavy/light) and *swe1Δ cdc55Δ* vs. *swe1Δ cdc55Δ* (heavy/light) were compared. For the overwhelming majority of peptides (97-99%), no significant variation in ratio was observed in the control set of experiments (see Appendix 6.1, Fig. 6.1).


A
Peptides with Full Cdk-consensus site - pS/TPxR/K
log2 ratio H/L G1 (A) - fold change
log2 ratio H/L G1 (B) - fold change
6
4
2
0
-2
-4
-6
-4
-2
0
2
4
6
Peptides with minimal Cdk-consensus site - pS/TP
log2 ratio H/L G1 (A) - fold change
log2 ratio H/L G1 (B) - fold change


B
Peptides with Full Cdk-consensus site - pS/TPxR/K
log2 ratio H/L S(A) - fold change
log2 ratio H/L S (B) - fold change
Peptides with minimal Cdk-consensus site - pS/TP
log2 ratio H/L S(A) - fold change
log2 ratio H/L S (B) - fold change

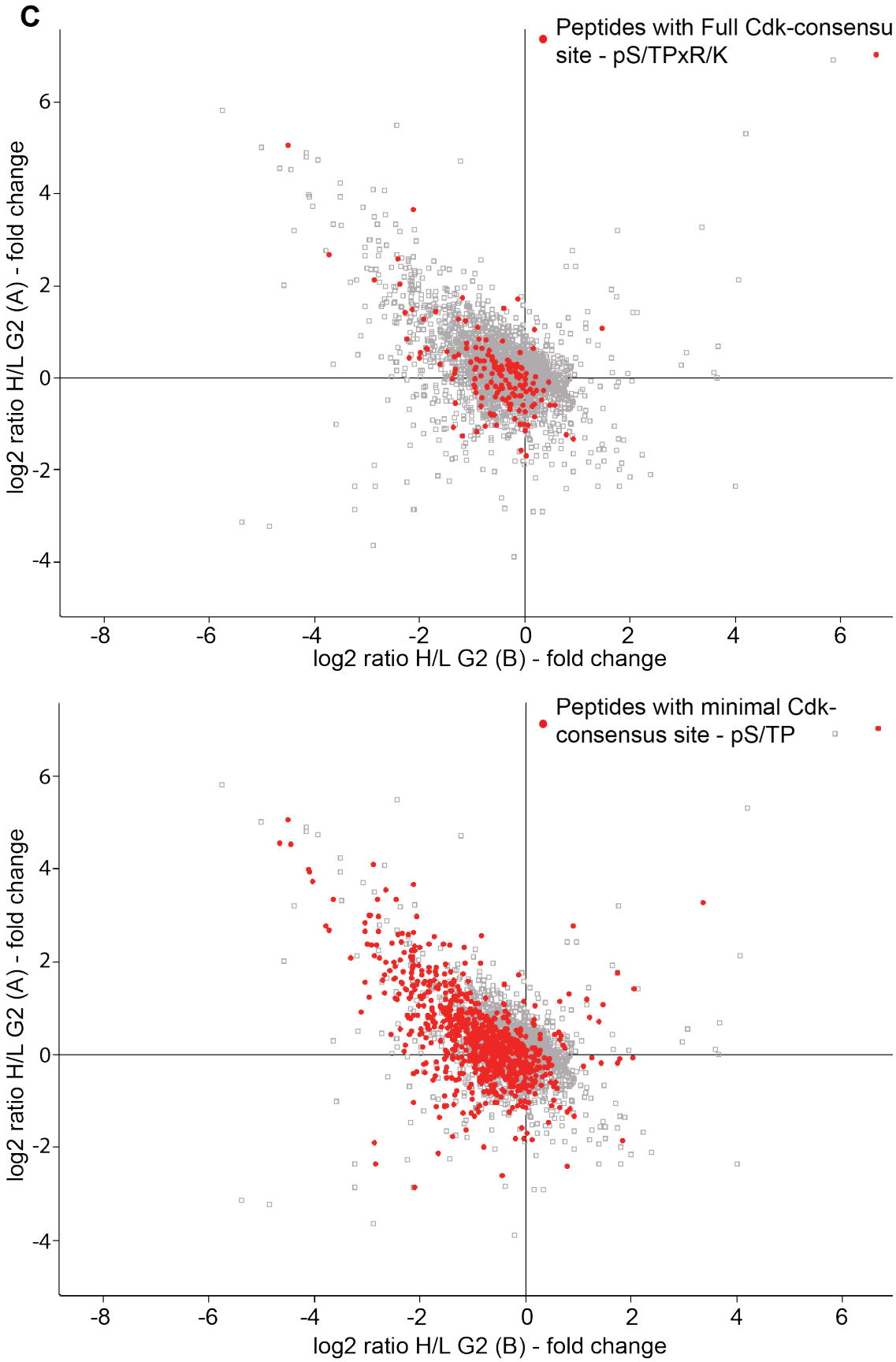



**Figure 4.10 – Cdk-motif containing phospho-peptides are enriched in the absence of PP2A$^{Cdc55}$ activity**

*(Figure 4.10 continued)* Correlation of the ratio of heavy/light Cdk-containing phospho-peptides between *swe1Δ cdc55Δ* and *swe1Δ* when the latter is "heavy" and the former is "light" (Experiment A) and vice-versa (Experiment B), in G1 (**A.**), S (**B.**) and G2 (**C.**). Phosphopeptides significantly enriched in the absence of phosphatase activity are situated in the top-left quadrant.

---

The specific identity of the Cdk-phosphorylated peptides is also of interest. The peptides were first examined to determine whether Cdk-phosphorylated peptides belonging to Acm1, Sli15 or Ndd1 had been detected in any of the experiments. One Cdk phosphorylated Acm1 peptide was identified in S and G2 but not in G1, with the phosphorylation site on S31, consensus pSPSK. It was not enriched in the absence of Cdc55 activity. In the case of Sli15, five Cdk phosphorylated sites were detected in total in the three experiments – two in G1, four in S and all five in G2, consistent with a gradual increase in phosphorylation of the protein as the cells progress through interphase. Four contained a minimal SP site, and one a full SPVR consensus site. Of these, one was slightly enriched in the absence of phosphatase activity, and the others had more stochastic behaviour – enriched in one repeat of the experiment and not the other, for instance. Finally, four Ndd1 phospho-peptides were identified, detected only in S phase and G2. Three of these were clearly enriched in *swe1Δ cdc55Δ* in both repeats of the experiment (Fig. 4.11). Of the four identified phospho-peptides, three were phosphorylated on a minimal TP consensus site and one on a minimal SP consensus site. Of the three phospho-peptides enriched in the absence of Cdc55, two were threonine phosphorylated.

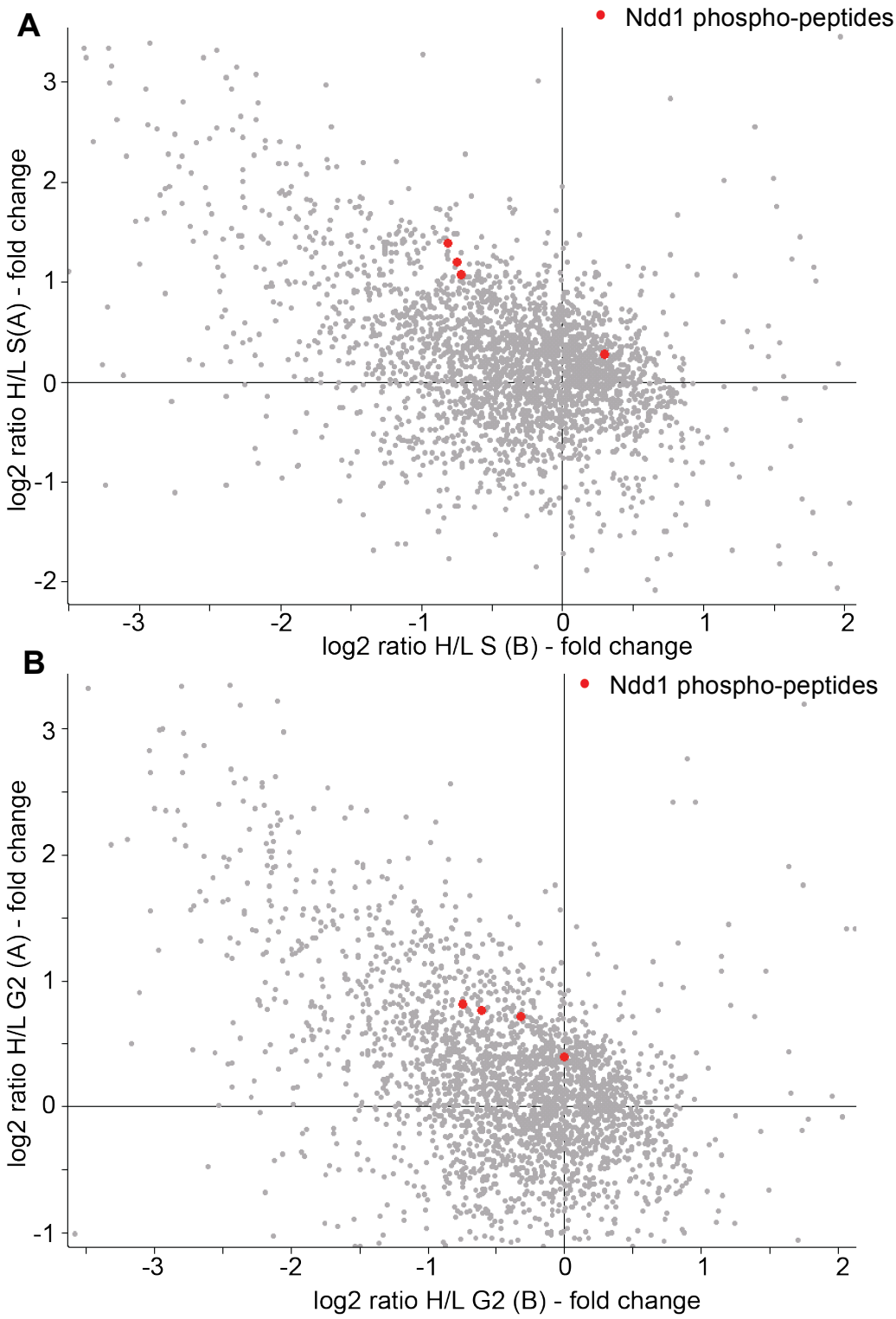



**Figure 4.11 – Cdk phosphorylation of Ndd1 on three residues is enriched in the absence of Cdc55**

Correlation of the ratio of heavy/light phospho-peptides between *swe1Δ cdc55Δ* and *swe1Δ* when the latter is "heavy" and the former is "light" and vice-versa, in S (**A.**) and G2 (**B.**). Phosphopeptides significantly enriched in the absence of phosphatase activity are situated in the top-left quadrant, and Ndd1 peptides are highlighted.

### 4.6.2 Cdk phosphorylation on threonines is enriched in the absence of PP2A$^{Cdc55}$ activity

In order to determine, in an unbiased manner, which types of phosphopeptides were enriched at each particular time point, a 2D annotation enrichment analysis was carried out (Cox and Mann, 2012). Using this method, respectively, 23 and, 31 and 32 different phosphopeptide "categories" were found to be enriched in the G1, S, and G2 samples, with a p value of <0.02. These categories ranged from specific kinase motifs, to peptides with a certain positive or negative charge, to protein binding motifs. Of specific interest are the different types of Cdk kinase motif. These were divided into 6 different and somewhat overlapping categories (all of which were identified in the 2D annotation enrichment analysis), not only separating minimal Cdk sites from full Cdk sites but also serine phosphorylation from threonine phosphorylation. These Cdk motifs are detailed in Table 4.3, as well as the total amount of phosphopeptides to be found within each category at each time point (added up over the six experiments).

| Category | Motif | Total Amount of Phosphopeptides | | |
|---|---|---|---|---|
| | | G1 | S | G2 |
| Full Cdk Site | pS/TPxR/k | 125 | 145 | 155 |
| Minimal Cdk Site | pS/TP | 914 | 846 | 851 |
| Full Cdk Site _pS | pSPxR/K | 106 | 121 | 127 |
| Full Cdk Site _pT | pTPxR/K | 19 | 24 | 28 |
| Minimal Cdk Site_pS | pSP | 691 | 640 | 643 |
| Minimal Cdk Site_pT | pTP | 223 | 206 | 208 |

**Table 4.3 – Cdk motif containing phosphopeptides are enriched throughout interphase**
Cdk motifs can be subdivided into full and minimal Cdk motifs, and whether the phosphorylated amino acid is a serine or a threonine. Full Cdk sites are much less abundant than minimal ones, particularly threonine-phosphorylated full sites.

From a superficial analysis of phosphopeptide abundance within each category, it can be concluded that serine phosphorylation by Cdk is more common than threonine phosphorylation, as has been previously observed (Kuilman et al., 2015). In fact, threonines make up 15-18% of phosphorylated full Cdk sites, and around 25% of minimal Cdk sites (Fig., 4.12).

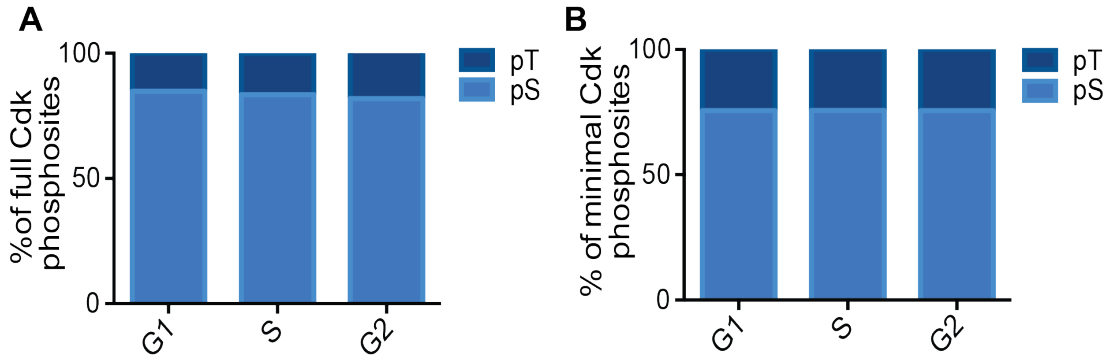


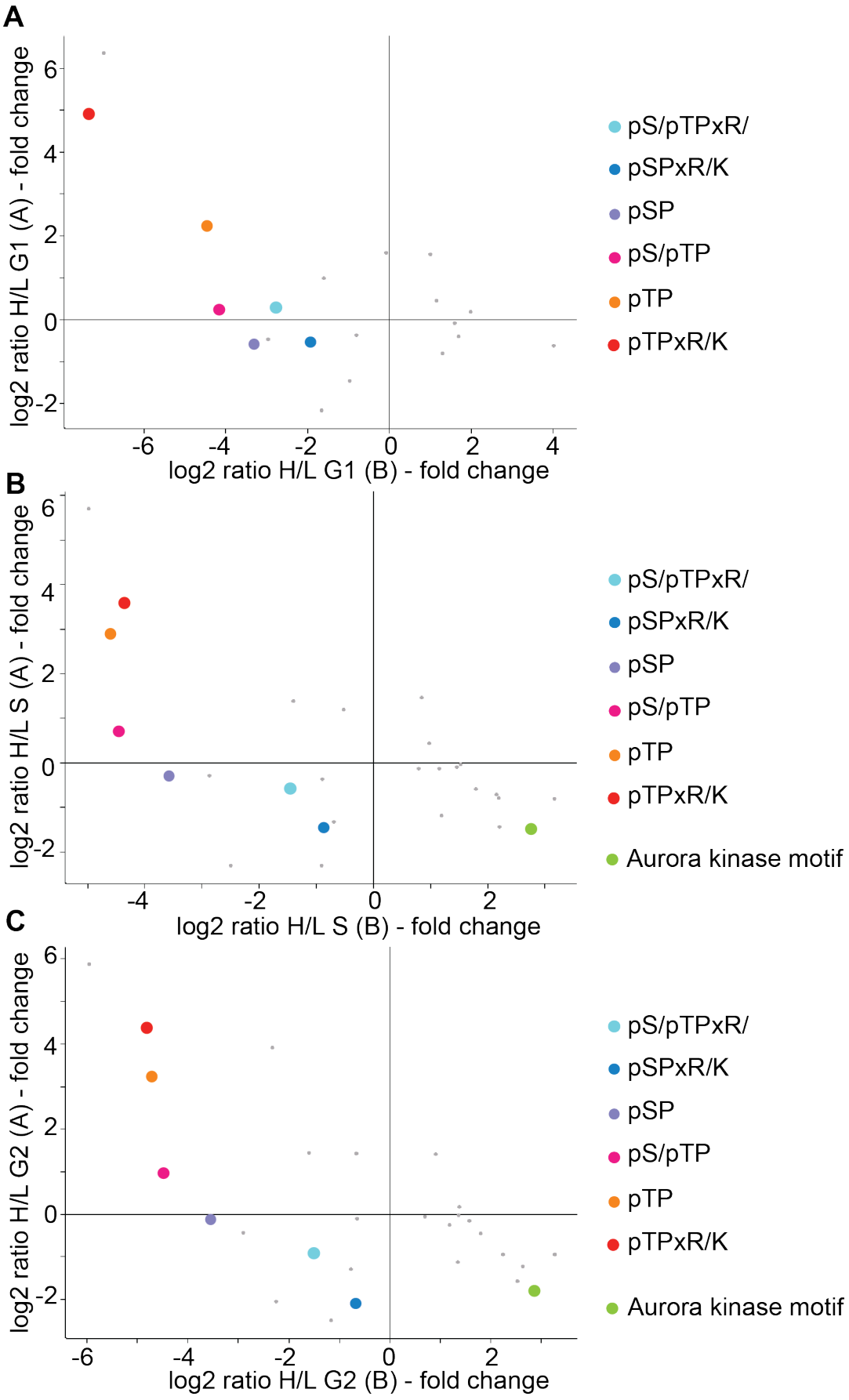

**Figure 4.12 – Threonines make up ~25% of total Cdk-phosphorylated sites**
Phospho-Threonines as a proportion of phosphorylated Cdk consensus sites, either full (**A.**) or minimal (**B.**), in G1, S and G2.

Next, we examined whether any in particular of these categories of Cdk sites was enriched in *swe1Δ cdc55Δ* compared to *swe1Δ* alone. Again, the ratio of heavy/light phosphopeptides in each category was correlated between the two strains, and plotted graphically as in Figure 4.10. Interestingly, Cdk substrate phosphorylation, although more so on minimal Cdk than full Cdk sites, appears mildly enriched in the absence of phosphatase activity. Strikingly, however, when Cdk phosphorylation is separated into serine versus threonine phosphorylation, this enrichment can in fact be accounted for by an increase in the phosphorylation of threonines. This is true for phosphopeptides with both full and minimal pTP Cdk phosphosites, even more strikingly so for the latter than the former (Fig. 4.13). Further, phosphorylation of threonines as a proportion of phosphorylation on full Cdk sites increases slightly as the cells progress through interphase (Fig. 4.12). To confirm the validity of this result, we examined whether in the control experiment, pTP phosphopeptides displayed any variation in ratio – this was not the case (see Appendix 6.1).

This indicates that PP2A$^{Cdc55}$ is preferentially working to dephosphorylate threonine-containing Cdk phospho-proteins during interphase, possibly having a higher affinity for full Cdk sites than minimal Cdk sites.

Of further interest is the fact that the Aurora kinase motif was also identified to be enriched in the SILAC experiment, although only in the S phase and G2 experiments. However, in contrast to what was observed with Cdk motifs, Aurora kinase motif containing peptides (of which respectively 49 and 50 were identified in S and G2) were highly enriched in the presence of PP2A$^{Cdc55}$ activity, not its absence.

**Figure 4.13 – Cdk phosphorylation on threonine, but not serine, residues is highly enriched in the absence of PP2A$^{Cdc55}$ activity**
(*following page)* 2D annotation enrichment analysis identifying enriched "categories" in the SILAC experiment, and a graphical annotation indicating whether any of these categories are more highly represented in the absence or presence of phosphatase activity, as in Figure 4.14, taking into account the two experimental repeats A and B. Categories enriched in the absence of PP2A$^{Cdc55}$ can be viewed in the top-left quadrant. The different categories of interest are indicated in colour.


A
log2 ratio H/L G1 (A) - fold change
log2 ratio H/L G1 (B) - fold change
B
log2 ratio H/L S (A) - fold change
log2 ratio H/L S (B) - fold change
C
log2 ratio H/L G2 (A) - fold change
log2 ratio H/L G2 (B) - fold change
pS/pTPxR/
pSPxR/K
pSP
pS/pTP
pTP
pTPxR/K
Aurora kinase motif

## 4.7 Roles for Cdc14 or PP1 in interphase remain unsubstantiated

As with PP2A$^{Cdc55}$, I studied the role of Cdc14 in interphase. I examined whether global Cdk phosphorylation levels were affected, as in Figure 4.4, and found no difference between the wild-type and a *cdc14-1* temperature sensitive strain at restrictive temperature, in synchronous cell cycle experiments. Further, I was unable to identify any interphase Cdk target whose phosphorylation was advanced in the absence of Cdc14 activity. Finally, elimination of Cdc14 activity in a G2-arrested state, using either *cdc28as-1* strains with varying concentrations of 1-NMPP1, or *clb1Δ clb3Δ clb4Δ clb2-ts cdc14-1* strains at restrictive temperature, did not have any effect.

Therefore, so far, I have been unable to demonstrate a role for Cdc14 in interphase in *S. cerevisiae*. The role of PP1 in interphase has yet to be examined.

## 4.8 Discussion and conclusions

### 4.8.1 PP2A$^{Cdc55}$ opposes phosphorylation of Cdk targets in interphase

From the results of the SILAC screen comparing Cdk phosphorylation in the presence and absence of PP2A$^{Cdc55}$ activity, as well as from using anti-phospho-Cdk site antibodies, we can conclude that PP2A$^{Cdc55}$ has a global effect on Cdk phosphorylation levels in interphase.

Further, I have also shown that the phosphorylation of three Cdk targets, Acm1, Sli1, and Ndd1, is advanced in the absence of PP2A$^{Cdc55}$ activity.

Interestingly, a recent publication has stated that the stability of Ndd1 is controlled by APC/C$^{Cdh1}$, with Ndd1 being a target for ubiquitin mediated proteasomal degradation in early interphase, preventing the too-early transcription of mitotic genes (Sajman et al., 2015). This explains my personal observations that Ndd1 is absent in G1. I have shown here that Acm1, the stability of which is controlled by phosphorylation of a specific residue by Cdk, appears earlier in the cell cycle in the absence of phosphatase

activity, presumably due to advanced phosphorylation of the protein on this residue. Acm1 being a stoichiometric inhibitor of APC/$C^{Cdh1}$, this then implies that the early appearance of Acm1 could in fact lead to earlier inactivation of the APC. Supporting this hypothesis, Ndd1 also appears earlier in the absence of Cdc55.

These results confirm our initial hypothesis, that Cdk-opposing phosphatase activity plays a role in interphase. However, our guess that phosphatases would be more important in G2 than in early interphase appears to be incorrect, at least when it comes to $PP2A^{Cdc55}$. Acm1 phosphorylation, normally occurring in late G1, seems to be advanced as much as Ndd1 phosphorylation, normally occurring in G2/M. Further, the results of the SILAC screen revealed a similar increase in Cdk phosphorylation levels whether the sample was taken in G1, S or G2, despite the G1 sample being taken before cells were released from α-factor, (thus pre-"Start"), with very low levels of Cdk activity.

These results, however, have to be tempered by the mention of the fact that the timing of phosphorylation of several more interphase Cdk targets including Mms4 and Src1, amongst others, was not affected by the absence of Cdc55.

### 4.8.2 $PP2A^{Cdc55}$ and Threonine dephosphorylation – a role beyond interphase?

In fact, perhaps the most striking result from the SILAC screen was not that global Cdk phosphorylation levels were elevated in the absence of Cdc55, but rather that this increase in levels can be explained solely by an increase in phosphorylation on threonines, and not on serines. This could also provide a possible explanation for the fact that some Cdk targets are affected by the absence of $PP2A^{Cdc55}$ and others not – those with threonine, rather than serine, Cdk sites phosphorylated *in vivo*. Is this the case for Acm1, Sli15 and Ndd1?

Strikingly, the sole Cdk site (out of 5) in Acm1 that has been shown to be important for its stability is T161, with the half-life of a non-phosphorylatable Acm1-T161A being significantly shorter than that of wild type Acm1 (Hall et al., 2008; Ostapenko et al., 2008).

Moreover, the sole Cdk site in Ndd1 so far confirmed to be phosphorylated by Cdk *in vivo*, and to be important for the interaction of this protein with its binding partner Fkh2 and thus binding to the DNA, is T319 (Reynolds et al., 2003; Darieva et al., 2003). However, this is just one of seventeen S/TP sites within the protein, 10 threonines (including T319) and 7 serines.

Which of the seventeen putative Cdk sites (14 SP's and 3 TP's) within Sli15 are phosphorylated *in vivo* has yet to be confirmed, although phosphorylation and dephosphorylation of 6 Cdk sites (5S/1T) within the microtubule binding domain of the protein is thought to be important for its function (Pereira and Schiebel, 2003). My observation that Sli15 is initially phosphorylated earlier in the absence of Cdc55 but reaches full phosphorylation at the same time as in the control could reflect the fact that initial phosphorylation may be occurring on the threonine residue but the bulk of phosphorylation occurs on serine residues.

On a side-note, and of further interest in discussing the advanced phosphorylation of Sli15, is the observation that Aurora kinase (Ipl1) seems to be less active when Cdc55 is absent from the cell, specifically in S phase and G2, but not G1 (Section 4.7.2 and Fig. 4.16). This indicates that $PP2A^{Cdc55}$ could be a positive regulator for this kinase at these cell cycle stages. In fact, Sli15 itself is a regulator of Ipl1; moreover it is thought to inhibit Ipl1's activity when phosphorylated by Cdk. It can be conjectured that increased phosphorylation of Sli15 by Cdk in the absence of Cdc55 could lead to hypo-activation of Ipl1. This provides a possible explanation for the decrease in Aurora-phosphorylated peptides observed by SILAC in the absence of Cdc55.

The fact that $PP2A^{Cdc55}$ seems to specifically oppose threonine phosphorylation leads us to ask whether it can only dephosphorylate threonines and not serines, a topic for further investigations. In fact, there is a precedent for this – Cdc14 has been shown to have a strong preference for serines over threonines (Bremmer et al., 2012).

Coincidentally, in this study by Bremmer *et al*, Cdc14 was shown to be unable to dephosphorylate T161 in Acm1 *in vitro*. Previously, I postulated that the timing of

phosphorylation of this same residue during interphase might be controlled by the ratio of $PP2A^{Cdc55}$/Cdk activity in the cells.

Further evidence for a preference for serines vs. threonines of Cdc14 came from substituting a threonine for a serine in an Acm1 Cdk-phosphorylated peptide (a 20-mer peptide phosphorylated on S31 in Acm1). *In vitro*, this resulted in a 3000-fold reduction in catalytic activity of Cdc14 towards the peptide (Bremmer et al., 2012). However, *in vivo*, Acm1 (and other phospho-threonine containing Cdk targets) are fully dephosphorylated during mitotic exit (Hall et al., 2008; Kuilman et al., 2015).These results have led to the assumption that another phosphatase aside from Cdc14 is responsible for dephosphorylating pT Cdk phosphosites during mitotic exit.

It is tempting to speculate that this is indeed the case, with the identity of this phosphatase being $PP2A^{Cdc55}$. Supporting a role for $PP2A^{Cdc55}$ in mitotic exit is the fact that anaphase is prolonged and cytokinesis is delayed in the absence of Cdc55 (Fig. 4.1). This does not fit with its known role as an inhibitor of mitotic exit (through opposing Net1 phosphorylation) – if this were the only function for $PP2A^{Cdc55}$ in mitotic exit it would be expected that absence of Cdc55 would shorten, rather than prolong, anaphase. The delay in mitotic exit in the *cdc55Δ* background has been previously reported, concomitant with a delay in Cdh1 dephosphorylation, and Cdc5 and Clb2 degradation, the latter of which I have also observed (Fig. 4.5) (Baro et al., 2013). I have also noted that high levels of Cdk phosphorylation persist throughout anaphase and mitotic exit in the absence of Cdc55 (Fig. 4.8). It has been proposed that $PP2A^{Cdc55}$ helps allow full activation of the MEN pathway in late anaphase by keeping Mob1 in a dephosphorylated and active state – which could (at least partly) explain the mitotic exit delay phenotype in *cdc55Δ* cells (Baro et al., 2013). My results open the possibility for a more direct contribution of Cdc55 to substrate dephosphorylation during mitotic exit.

Given the delay in Clb2 and Cdc5 degradation, both of which are $APC^{Cdh1}$ substrates, it has been suggested that activation of $APC^{Cdh1}$ is compromised in this case, especially given that phosphorylation and dephosphorylation of Cdh1 on Cdk sites (by Cdk/

Cdc14) is thought to control its activity (Morgan, 2007; Baro et al., 2013). On the one hand, it is possible that a delay in APC activation in *cdc55Δ* can be explained by compromised MEN activation (and thus compromised Cdc14 release) – however this is counterintuitive, as in the absence of Cdc55, Cdc14 is more, rather than less active. Another possibility is that $PP2A^{Cdc55}$ can directly regulate Cdh1 phosphorylation. Further, it is conceivable that $PP2A^{Cdc55}$ also regulates the phosphorylation state (and thus stability) of Acm1 at mitotic exit, as it does in interphase (Jaspersen, Charles and Morgan, 1999; Hall, Warren and Borchers, 2004). A small delay in Acm1 destruction was observed in my hands in the absence of Cdc55; whether this actually influences the timing of $APC^{Cdh1}$ activation remains to be examined.

### 4.8.3 Future perspectives

The role of Cdk-counteracting phosphatases in interphase is still far from clear. Notably, although I have shown that $PP2A^{Cdc55}$ opposes phosphorylation of Cdk targets in interphase, I have yet to find a phenotypic consequence for the absence of phosphatase activity. As such, cells lacking $PP2A^{Cdc55}$ activity do not progress through interphase any faster than control cells, or manage to successfully escape from a G2 arrest caused by low Cdk activity.

In order to verify that $PP2A^{Cdc55}$ activity in interphase is important *in vivo*, a careful analysis of the phenotype of cells lacking $PP2A^{Cdc55}$ activity will need to be carried out. It will be interesting to determine whether lack of Cdc55 could rescue milder cell cycle progression phenotypes due to reduced Cdk activity, such is the case with some single cyclin deletions, or the temperature sensitive G2/M delay caused by the *cdc28-1N* allele.

Many Cdk targets are not affected by the absence of $PP2A^{Cdc55}$, and this phosphatase seems to mainly affect threonine phosphorylation. This makes it unlikely that it should be the main, or the only, Cdk-opposing phosphatase acting during interphase. This is particularly true if our hypothesis is correct and Cdk-opposing phosphatase activity is a necessary part of the quantitative model for the cell cycle.

It can be speculated that $PP2A^{Cdc55}$ is not acting alone in opposing Cdk phosphorylation in interphase. Although functions for PP1 and/or Cdc14 have not yet been validated, we cannot rule out that these phosphatases also have a role in interphase. Perhaps all three phosphatases, acting upon different targets, contribute towards the regulation of the timing of mitotic progression, playing their particular part in the quantitative model for the cell cycle. Much work remains to be done in investigating whether this is the case.

Firstly, it will be important to investigate whether PP1 has any role in interphase. To this end, interphase Cdk phosphorylation could be examined in a *glc7-ts* mutant background, using the same strategies as those in the earlier sections of this chapter – looking at specific Cdk targets and global interphase Cdk phosphorylation by western blot, possibly followed by SILAC if the results are promising.

It also seems likely that the three serine/threonine phosphatases, having some measure of overlapping activity (and therefore possibly acting towards the same targets) could compensate for each other. As such, investigating the effects of double phosphatase mutants (for instance, comparing *swe1Δ*, *swe1Δ cdc55Δ, cdc14-1 swe1Δ* and *cdc14-1 swe1Δ cdc55Δ* strains) to determine whether removing phosphatases together has additive effects, will be important. The timing of progression through interphase should be compared in these backgrounds, as well as levels of Cdk phosphorylation (using anti-phospho-Cdk antibodies). If results are promising, SILAC experiments can also be envisaged.

# Chapter 5. Final Discussion

In this thesis, I have provided new insights into the roles and regulation of Cdk-counteracting phosphatases, both during mitotic exit and in interphase.

## 5.1 Phosphatases are a vital component of the quantitative model for the cell cycle

I hope to have furthered our understanding of the quantitative mechanisms controlling the cell cycle.

On the one hand, I have reinforced the idea that systems-level feedback plays an important role in cell cycle control, using budding yeast mitotic exit as an example. The fact that Cdc14 release is so stringently regulated, on so many levels, is yet another indication that the balance of kinase/phosphatase activities during this stage of the cell cycle is crucial for the timing of Cdk substrate dephosphorylation and thus cell cycle progression. The presence of the positive feedback loop for Cdc14 release in early anaphase allows for rapid amplification of Cdc14 activity, compensating for the adverse effect of Cdk downregulation on maintaining Net1 phosphorylation (and therefore Cdc14 release).

More generally, the importance of feedback loops and ultra-sensitive mechanisms in cell cycle control, causing transitions between states to become both sharpened and irreversible, is becoming ever clearer (López-Avilés et al., 2009; Kapuy et al., 2009; Domingo-Sananes, Kapuy, Hunt and Novak, 2011; Jr, 2013; Santos and Ferrell, 2008). In particular, it has been highlighted in recent years through the use of computational modelling.

On the other hand, I have established that the ratio of kinase/phosphatase activity is also a vital determinant for the timing of substrate phosphorylation during interphase. This is not in fact surprising, as the existence of this phosphatase activity has been postulated and even become an accepted part of cell cycle models in recent years (Barr,

Elliott and Gruneberg, 2011; Jr, 2013; Domingo-Sananes et al., 2011). However, this is the first formal demonstration that PP2A directly opposes phosphorylation of specific Cdk targets in interphase, independently of its role in regulating the activity of Cdk.

Again, it will be important to understand how ordered target phosphorylation is achieved, from a quantitative point of view. Is it that phosphatase activity is constant during interphase and kinase activity is increasing, with some substrates more sensitive to the kinase but equally sensitive to the phosphatase, that causes the substrates to be phosphorylated in an orderly manner? If this were the case, the role of phosphatase activity would simply be to improve the time-resolution of phosphorylation; spreading substrate phosphorylation over a longer time-period than could be achieved with the kinase acting alone. Alternatively, it's also possible that certain substrates are better for the kinase than for the phosphatase (and vice-versa), as during mitotic exit? In either of these cases, the biochemical basis behind substrate preference, either by the kinase or phosphatase, that causes them to be phosphorylated at a specific time, is an important area for further study.

## 5.2 Future perspectives – moving on from budding yeast to other model organisms

Finally, deepening our understanding of the roles of Cdk-opposing phosphatase activity in higher eukaryotes must remain our ultimate goal. We need to understand exactly how mitotic exit is controlled in other organisms, and validate the role of Cdk-opposing phosphatases at this cell cycle stage. Indeed, it will be interesting to determine whether a preference for threonines over serines is specific to budding yeast $PP2A^{Cdc55}$ (and in fact, within budding yeast, specific to this isoform of the enzyme), or conserved in different species.

Given how stringently Cdc14 is regulated in budding yeast, we can reason that in other organisms, the mechanisms of regulation of phosphatase activity, restricting their activity to particular cell cycle locations and particular cell cycle times, will also be conserved. Recent studies are already giving indications as to how Cdk-counteracting

phosphatase activity is regulated in other systems, although it seems the more we understand, the more levels of regulation are being uncovered (Grallert et al., 2015a; Cundell, Bastos, Zhang and Holder, 2013; Glover, 2012; Grallert et al., 2015b; Schmitz et al., 2010).

Although it seems logical that the quantitative model for cell cycle progression should hold true in more complex organisms, and some clues have indicated that the phosphorylation of at least a subset or proteins depends on the kinase/phosphatase ratio (Wolf, Wandke, Isenberg and Geley, 2006; Garriga et al., 2004), this has yet to be validated and remains a crucial area for investigation. Working towards this, some insights into the mechanistic basis for phosphatase specificity in early mitotic exit in mammalian cells have been recently provided (McCloy et al., 2015).

Similarly, we need to move on from mathematical modelling of the (relatively simple) yeast or *X. laevis* embryonic cell cycles to modelling that of mammalian cells – incorporating phosphatase activity as a key determinant of the timing of cell cycle events and transitions in both systems.

In a wider context, the ultimate aim of elucidating the mechanisms behind the control of cell division is to understand how perturbations in the human cell cycle machinery lead to the development of cancer. Accurate predictive models of the cell cycle could help in the development of new ways to target the weaknesses of cancer cells, or in the restoration of cell cycle control.

# Chapter 6. Appendix

## 6.1 SILAC screen (2) – additional data and controls

As discussed in sections 4.5-6, a SILAC screen was carried out in order to compare Cdk phosphorylation at different stages in interphase between *swe1Δ* and *swe1Δ cdc55Δ* strains. As a quality control, experiments were performed in which at each time point (G1/S/G2), *swe1Δ* vs. *swe1Δ* (heavy/light) and *swe1Δ cdc55Δ* vs. *swe1Δ cdc55Δ* (heavy/light) were compared. No significant variation in phospho-peptide ratios was noted in any of the experimental samples. This includes all Cdk phospho-peptides, whether serine or threonine phosphorylated. Indeed, in each experiment, fewer than 5% (and in general closer to 1%) of Cdk phosphorylated phospho-peptides were enriched in either the heavy or light cultures (Fig. 6.1). Importantly, as a control for the results of the 2D annotation analysis, TP phosphopeptides are not enriched in the heavy/light in any of the control experiments.

By comparison, looking at the scatter plots for each individual sample mix, significant variations in phospho-peptide ratios (reflecting the fact that Cdk phospho-peptides are enriched in the absence of Cdc55, and more specifically TP phosphopeptides, as detailed in Fig. 4.13) are clearly noticeable. In fact, in each of these 6 samples, respectively between 10 and 25% of Cdk phosphopeptides are enriched in *swe1Δ cdc55Δ* vs. *swe1 Δ*, as compared to the 0-4% in *swe1 Δ* vs. *swe1Δ cdc55Δ* (Fig. 6.2).

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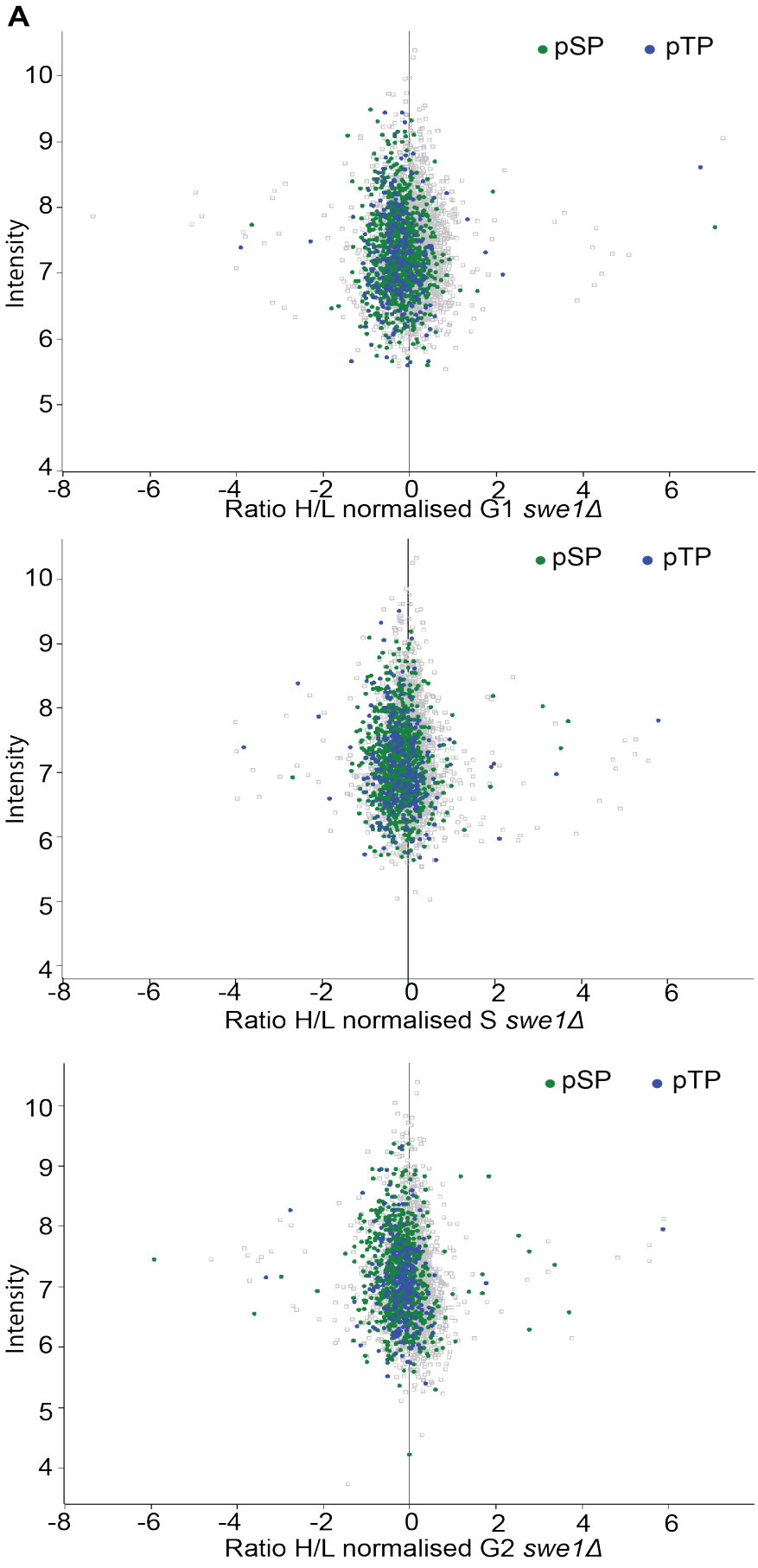
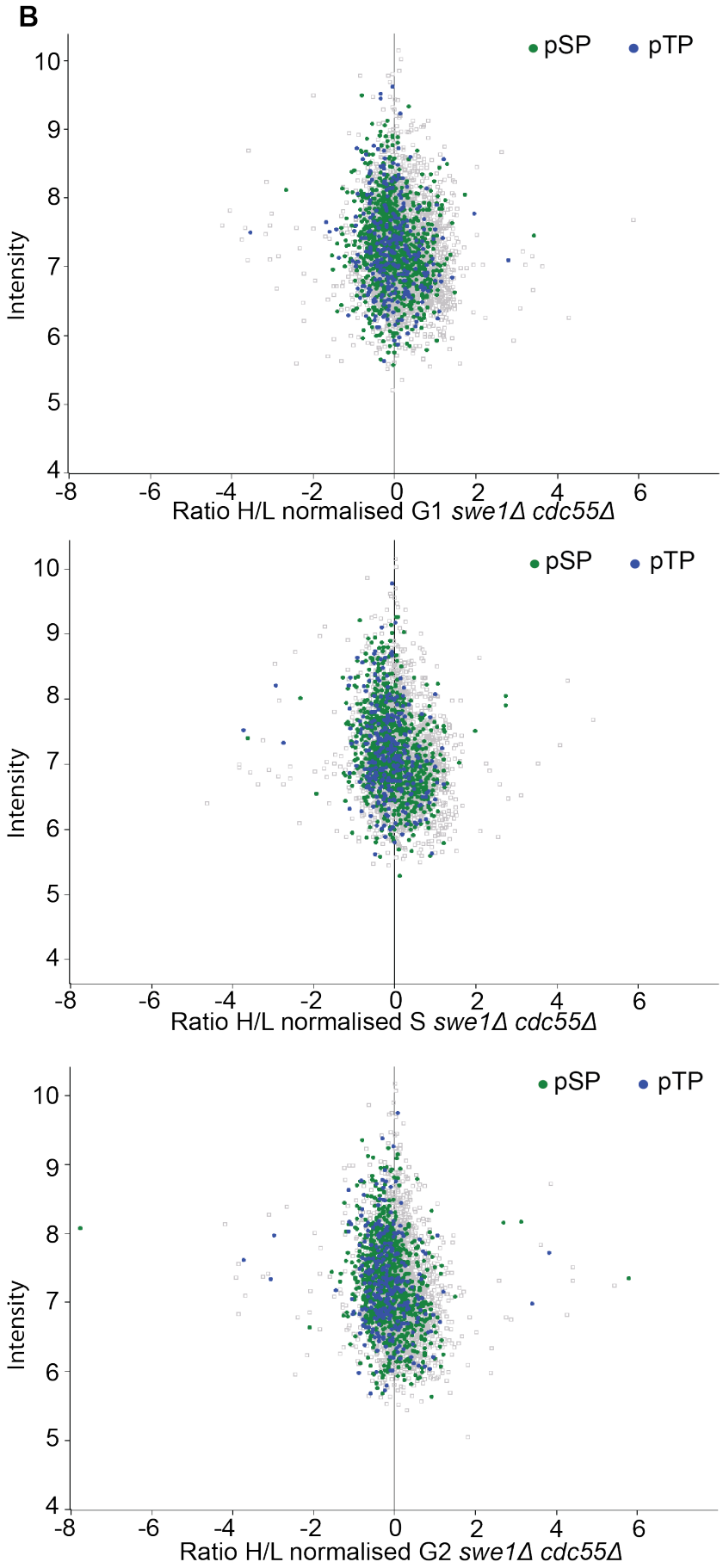
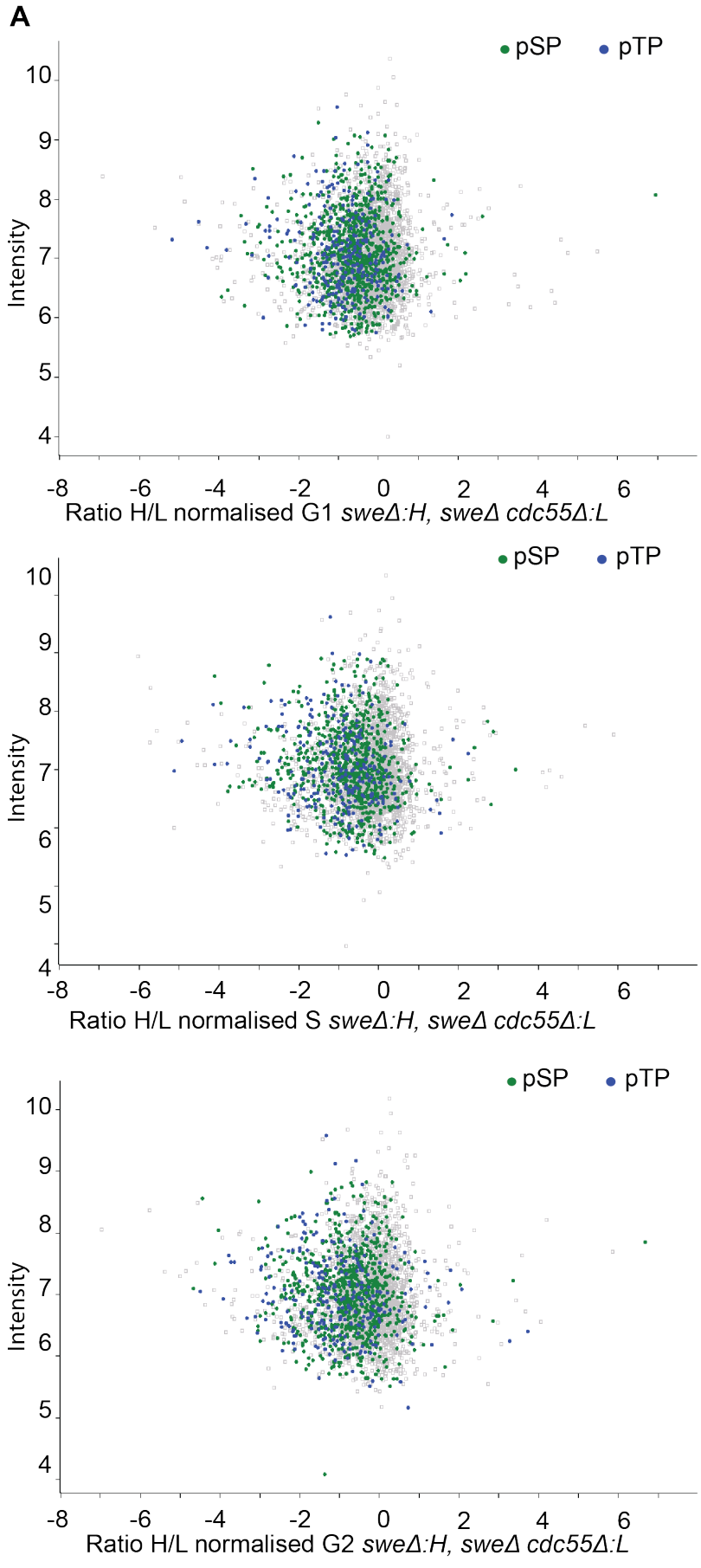
**Figure 6.1 – SILAC quality control, *swe1Δ* vs. *swe1Δ* (H/L) and *swe1Δ cdc55Δ* vs. *swe1Δ cdc55Δ* (H/L)**
*(following page)* Ratio of heavy/light phospho-peptides versus peptide intensity, in G1, S and G2 in either *swe1* versus *swe1* (heavy/light) (**A.**) or *swe1Δ cdc55Δ* versus *swe1Δ cdc55Δ* **(B.).** Cdk phospho-peptides are indicated in green and blue, separated respectively into serines and threonines.


A
pSP
pTP
Intensity
Ratio H/L normalised G1 swe1Δ
Ratio H/L normalised S swe1Δ
Ratio H/L normalised G2 swe1Δ


B
pSP
pTP
Intensity
Ratio H/L normalised G1 swe1Δ cdc55Δ
Ratio H/L normalised S swe1Δ cdc55Δ
Ratio H/L normalised G2 swe1Δ cdc55Δ


A
pSP
pTP
Intensity
10
9
8
7
6
5
4
-8
-6
-4
-2
0
2
4
6
Ratio H/L normalised G1 sweΔ:H, sweΔ cdc55Δ:L
pSP
pTP
Intensity
Ratio H/L normalised S sweΔ:H, sweΔ cdc55Δ:L
pSP
pTP
Intensity
Ratio H/L normalised G2 sweΔ:H, sweΔ cdc55Δ:L

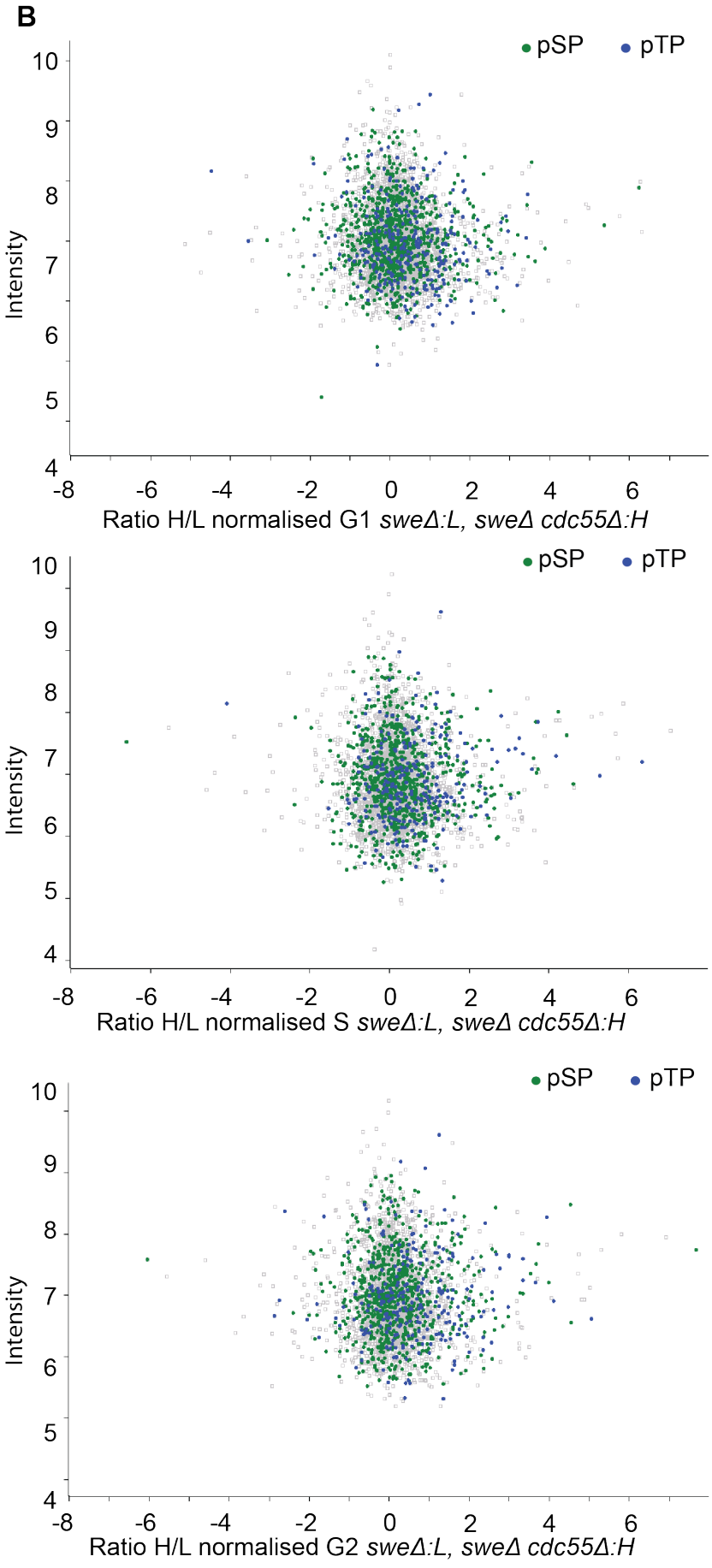

B
pSP
pTP
Intensity
Ratio H/L normalised G1 sweΔ:L, sweΔ cdc55Δ:H
Ratio H/L normalised S sweΔ:L, sweΔ cdc55Δ:H
Ratio H/L normalised G2 sweΔ:L, sweΔ cdc55Δ:H

**Figure 6.2 – Phospho-peptide ratios in each individual SILAC mix, *swe1 Δ* vs. *swe1Δ cdc55Δ***
*(previous two pages)* Ratio of heavy/light phospho-peptides versus peptide intensity, in G1, S and G2 in either *swe1* "heavy" versus *swe1Δ cdc55Δ* "light" (**A.**) or vice-versa **(B.).** Cdk phospho-peptides are indicated in green and blue, separated respectively into serines and threonines.

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